US009670527B2

(12) **United States Patent**
Vogelstein et al.

(10) **Patent No.: US 9,670,527 B2**
(45) **Date of Patent: Jun. 6, 2017**

(54) **ENRICHMENT OF NUCLEIC ACIDS BY COMPLIMENTARY CAPTURE**

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(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 252 days.

(21) Appl. No.: **14/130,007**

(22) PCT Filed: **Jun. 28, 2012**

(86) PCT No.: **PCT/US2012/044634**

§ 371 (c)(1),
(2), (4) Date: **Jan. 29, 2014**

(87) PCT Pub. No.: **WO2013/003585**

PCT Pub. Date: **Jan. 3, 2013**

(65) **Prior Publication Data**

US 2014/0154683 A1 Jun. 5, 2014

Related U.S. Application Data

(60) Provisional application No. 61/502,622, filed on Jun. 29, 2011.

(51) **Int. Cl.**
C12Q 1/68 (2006.01)

(52) **U.S. Cl.**
CPC ***C12Q 1/6806*** (2013.01)

(58) **Field of Classification Search**
None
See application file for complete search history.

(56) **References Cited**

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(57) **ABSTRACT**

Assays can be used to detect mutations found in neoplasms of the pancreas, as well as for other neoplasms and other uses. Nucleic acids can be captured from body fluids such as cyst fluids. Thousands of oligonucleotides can be synthesized in parallel, amplified and ligated together. The ligated products can be further amplified. The amplified, ligated products are used to capture complementary DNA sequences, which can be analyzed, for example by massively parallel sequencing.

22 Claims, 17 Drawing Sheets

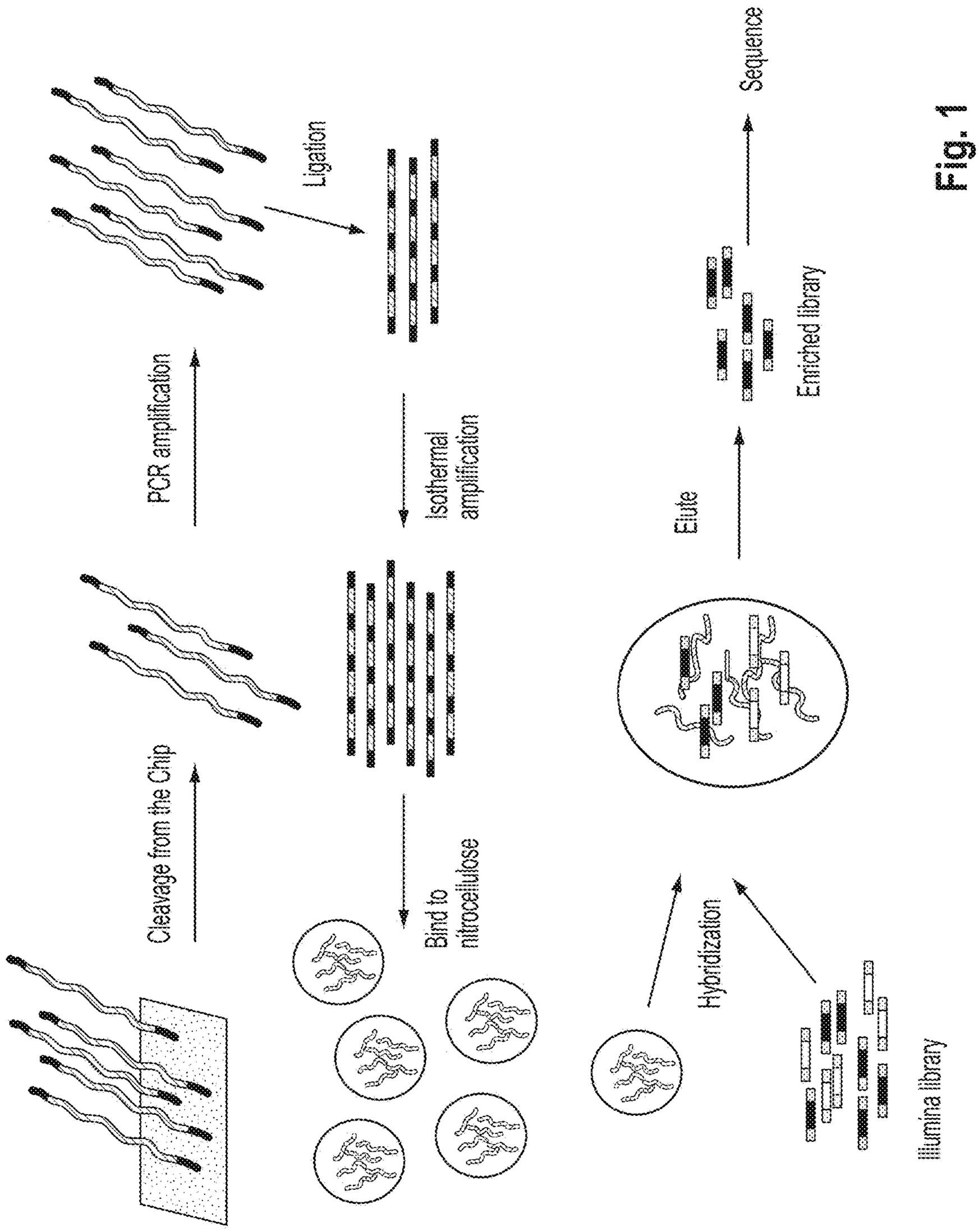

Ligation
PCR amplification
Isothermal amplification
Cleavage from the Chip
Bind to nitrocellulose
Elute
Hybridization
Sequence
Enriched library
Illumina library

Fig. 1

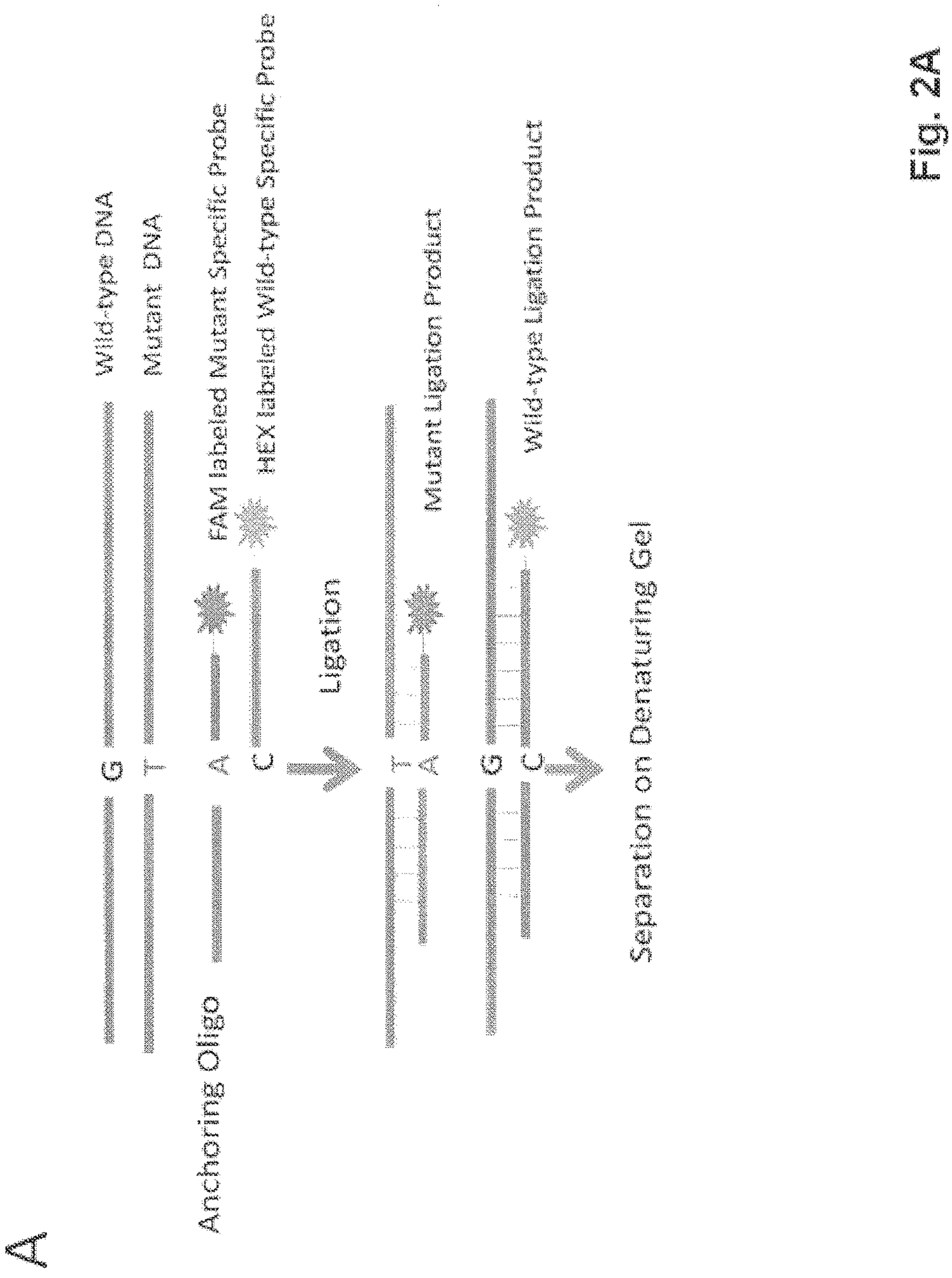
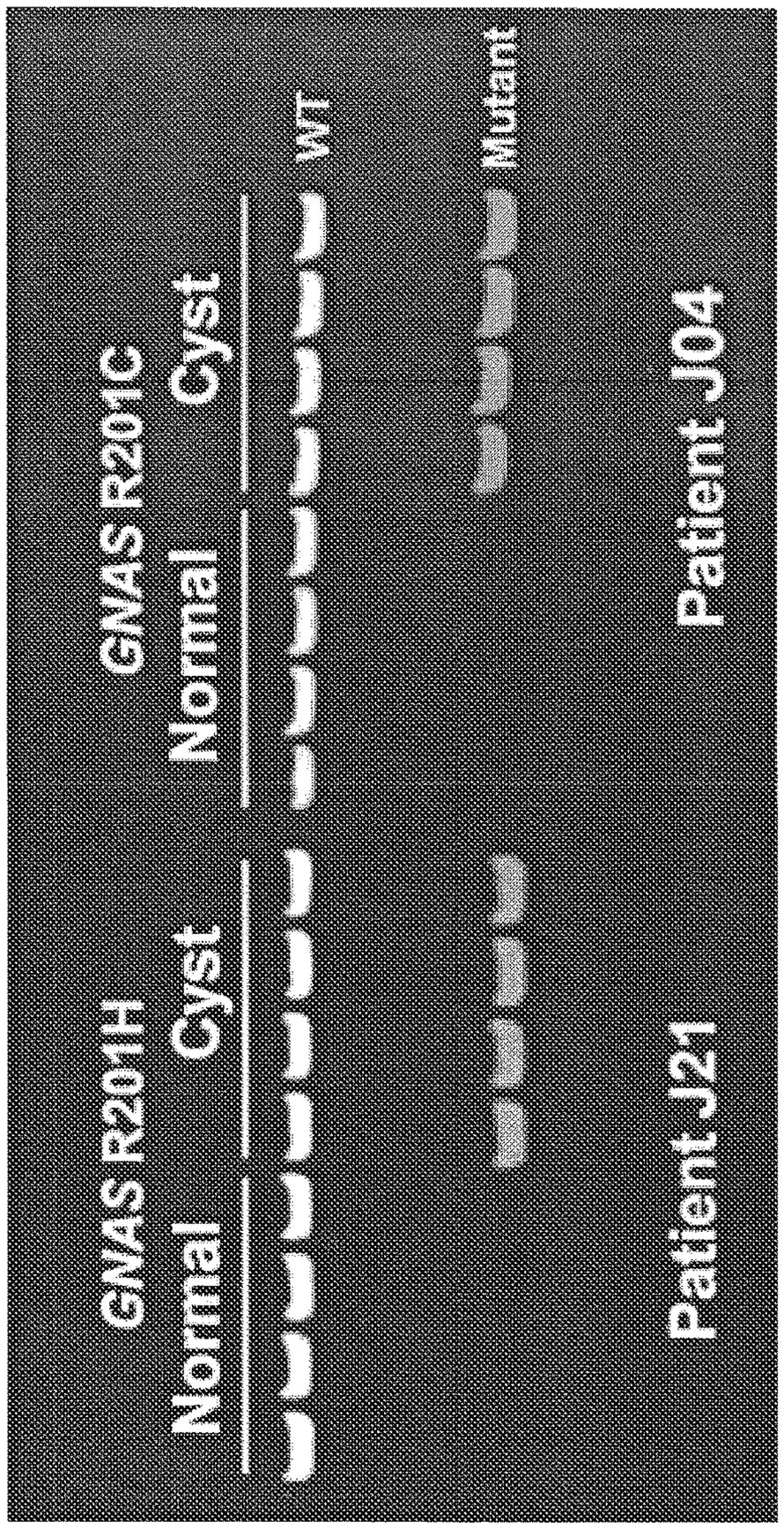

A
Anchoring Oligo
Wild-type DNA
Mutant DNA
FAM labeled Mutant Specific Probe
HEX labeled Wild-type Specific Probe
Ligation
Mutant Ligation Product
Wild-type Ligation Product
Separation on Denaturing Gel

Fig. 2A


GNAS R201C
Normal
Cyst
WT
Mutant
GNAS R201H
Normal
Cyst
Patient J04
Patient J21

B

Fig. 2B

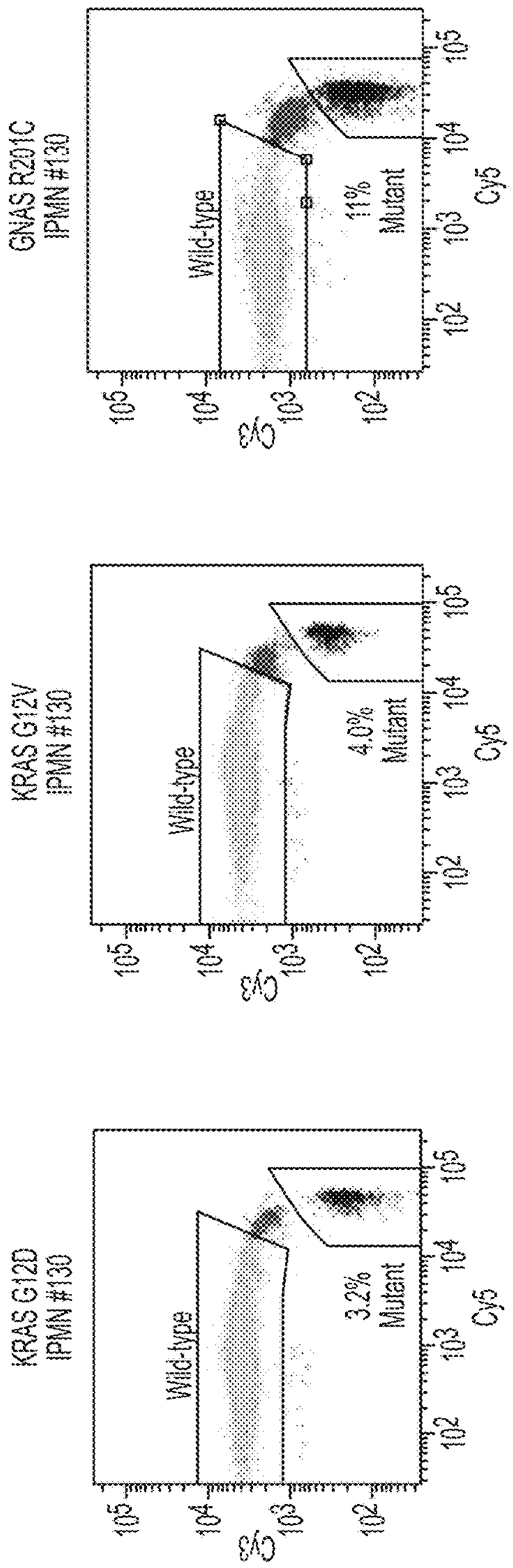

GNAS R201C
IPMN #130
Wild-type
11%
Mutant
Cy3
Cy5
NORMAL CELLS
0%
Mutant
KRAS G12V
IPMN #130
4.0%
Mutant
KRAS G12D
IPMN #130
3.2%
Mutant
10^2
10^3
10^4
10^5

Fig. 3

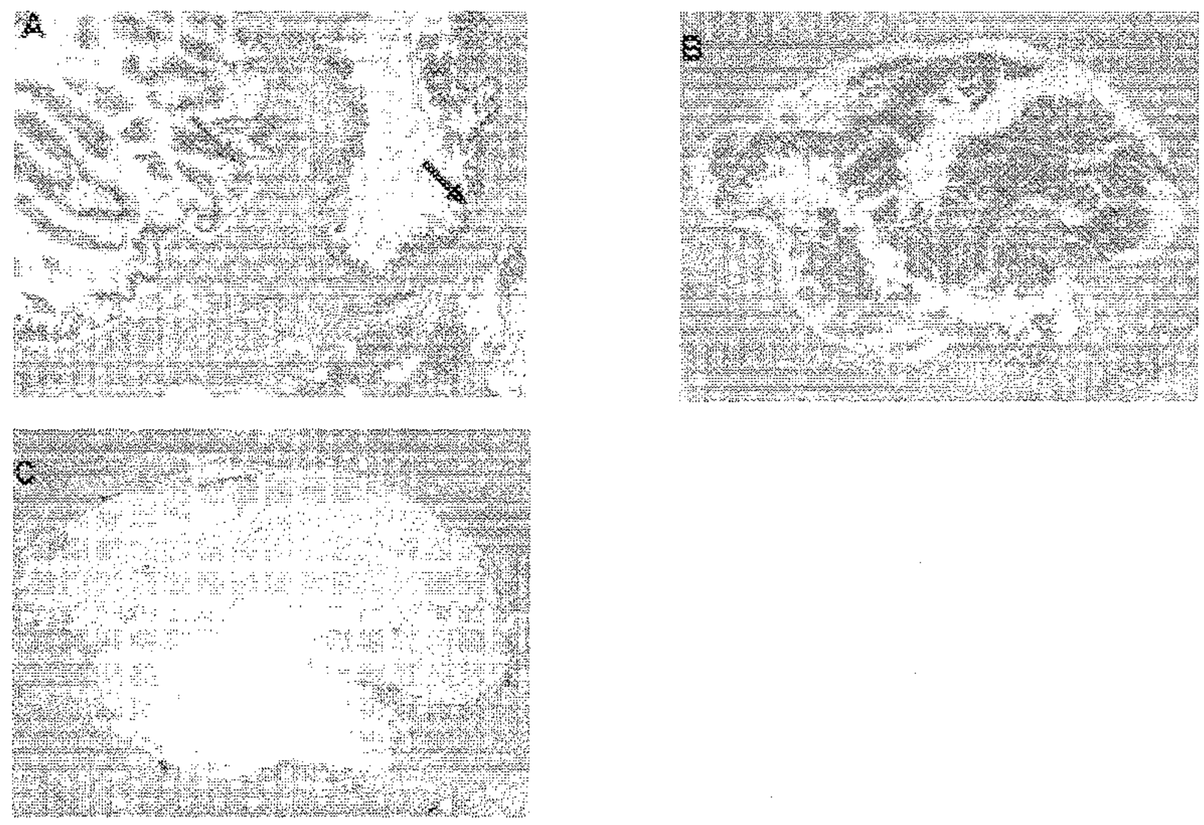


Fig. 4

Table S1. Genes analyzed by massively parallel sequencing in IPMN cyst fluids.

Gene symbol	Accession Number	Oncogene or Tumor Suppressor Gene
ABL1	X16416	Oncogene
ARHGAP29	NM_004815.2	Suppressor Gene
AKT1	NM_005163	Oncogene
ALK	NM_004304	Oncogene
JARID1C	NM_004187.1	Suppressor Gene
APC	NM_000038	Suppressor Gene
ATM	NM_000051	Suppressor Gene
UBR4	NM_020765.1	Suppressor Gene
BRAF	NM_004333	Oncogene
BRCA1	NM_007294.1	Suppressor Gene
BRCA2	NM_000059.1	Suppressor Gene
CBL	NM_005188.1	Oncogene
CDC73	NM_024529.3	Suppressor Gene
CDH1	NM_004360.2	Suppressor Gene
CDKN2A	NM_000077	Suppressor Gene
CEBPA	NM_004364.2	Suppressor Gene
CSF1R	NM_005211	Oncogene
CTNNA1	NM_001903.2	Suppressor Gene
CTNNB1	NM_001904	Oncogene
ATR	NM_001184	Suppressor Gene
CYLD	NM_015247.1	Suppressor Gene
LRRK2	SU_LRRK2	Suppressor Gene
KIAA1409	ENST00000256339	Suppressor Gene
SPTAN1	ENST00000372731	Suppressor Gene
ATRX	NM_138271.1	Suppressor Gene
SPEG	SU_SPEG	Suppressor Gene
MAST4	SU_MAST4	Suppressor Gene
DPYSL4	ENST00000338492	Oncogene
EGFL6	NM_015507.2	Oncogene
EGFR	NM_005228	Oncogene
WNK2	SU_WNK2	Suppressor Gene
CAD	NM_004341.2	Suppressor Gene
SORL1	ENST00000260197	Suppressor Gene
NUP214	NM_005085.2	Suppressor Gene
ERBB2	NM_004448	Oncogene
TECTA	ENST00000392793	Suppressor Gene
ADAMTS20	NM_025003.2	Suppressor Gene
TRIP11	ENST00000267622	Suppressor Gene
FAM123B	NM_152424.1	Suppressor Gene
FBXW7	NM_033632.1	Suppressor Gene
TAF1	NM_138923	Suppressor Gene
FGFR3	NM_000142	Oncogene
FLT3	Z26652	Oncogene

FIG. 5A

COL14A1	NM_021110.1	Suppressor Gene
FOXL2	NM_023067.2	Oncogene
NUP98	NM_016320.2	Suppressor Gene
GATA1	NM_002049.2	Suppressor Gene
CDC42BPB	NM_006035	Suppressor Gene
LTBP1	NM_206943.1	Suppressor Gene
TAF1L	NM_153809	Suppressor Gene
GNAQ	NM_002072.2	Oncogene
GNAS	NM_000516.3	Oncogene
ITSN2	NM_006277.1	Suppressor Gene
N4BP2	NM_018177.2	Suppressor Gene
JARID1A	NM_005056.1	Suppressor Gene
DEPDC2	NM_024870.2	Suppressor Gene
HNF1A	NM_000545.3	Suppressor Gene
HRAS	NM_005343	Oncogene
IDH1	NM_005896.2	Oncogene
IDH2	NM_002168.2	Oncogene
GLI3	NM_000168.2	Suppressor Gene
CENTD3	NM_022481.4	Suppressor Gene
BAZ1A	NM_013448.2	Suppressor Gene
MAP4K4	NM_145686	Suppressor Gene
COL1A1	ENST00000225964	Suppressor Gene
ASXL1	ENST00000358956	Suppressor Gene
JAK2	NM_004972	Oncogene
JAK3	NM_000215	Oncogene
ROCK2	NM_004850	Suppressor Gene
ROCK1	NM_005406	Suppressor Gene
IKBKAP	NM_003640.2	Suppressor Gene
KIT	NM_000222	Oncogene
IGF1R	NM_000875	Suppressor Gene
KRAS	NM_004985	Oncogene
STK36	NM_015690	Suppressor Gene
RAD50	NM_133482.1	Suppressor Gene
MAP3K6	NM_004672	Suppressor Gene
PER1	ENST00000317276	Suppressor Gene
WNK4	NM_032387	Suppressor Gene
MAP2K4	NM_003010	Suppressor Gene
ADAMTS18	NM_199355.1	Suppressor Gene
MGA	XM_031689.7	Suppressor Gene
ABL2	NM_005158	Suppressor Gene
TSC1	NM_000368.2	Suppressor Gene
MEN1	ENST00000312049	Suppressor Gene
MET	NM_000245	Oncogene
TNKS2	AF264912.1	Suppressor Gene
TNK2	NM_005781	Suppressor Gene
TRIM33	NM_015906	Suppressor Gene

FIG. 5B

MLH1	NM_000249.2	Suppressor Gene
ULK2	NM_014683	Suppressor Gene
GUCY2F	NM_001522	Suppressor Gene
HDAC4	NM_006037.2	Suppressor Gene
MPL	NM_005373.1	Oncogene
MSH2	NM_000251.1	Suppressor Gene
MSH6	NM_000179.1	Suppressor Gene
ERN2	NM_033266.1	Suppressor Gene
USP24	XM_371254.3	Suppressor Gene
NF1	ENST00000358273	Suppressor Gene
NF2	NM_000268.2	Suppressor Gene
NFKB1	NM_003998.2	Suppressor Gene
EPHB1	NM_004441	Suppressor Gene
NOTCH1	NM_017617.2	Suppressor Gene
NOTCH2	NM_024408.2	Suppressor Gene
NPM1	NM_002520.4	Suppressor Gene
NRAS	NM_002524	Oncogene
PHF14	NM_001007157.1	Suppressor Gene
ROR2	NM_004560	Suppressor Gene
TNPO1	NM_002270.2	Suppressor Gene
PDGFRA	NM_006206	Oncogene
AXL	NM_001699	Suppressor Gene
PRKD2	NM_016457	Suppressor Gene
TTK	NM_003318	Suppressor Gene
PIK3CA	NM_006218.1	Oncogene
TNNI3K	NM_015978	Suppressor Gene
PRKAR1A	NM_212472.1	Suppressor Gene
VEPH1	ENST00000392832	Suppressor Gene
HIF1A	NM_001530.2	Suppressor Gene
PTCH1	NM_000264.2	Suppressor Gene
PTEN	NM_000314.4	Suppressor Gene
PTPN11	NM_002834.3	Oncogene
PTPRC	NM_002838.2	Oncogene
RPS6KA2	NM_021135	Suppressor Gene
BRD2	NM_005104	Suppressor Gene
ITGB3	NM_000212.2	Suppressor Gene
RB1	NM_000321	Suppressor Gene
RET	NM_020975	Oncogene
ADAM29	NM_014269.2	Suppressor Gene
ANAPC5	NM_016237.3	Suppressor Gene
ITGB2	NM_000211.1	Suppressor Gene
CHUK	NM_001278	Suppressor Gene
TCF12	NM_207037.1	Suppressor Gene
PDZRN4	NM_013377.2	Suppressor Gene
RUNX1	ENST00000300305	Suppressor Gene
SETD2	ENST00000330022	Suppressor Gene

FIG. 5C

SMAD2	NM_005901.3	Suppressor Gene
SMAD4	NM_005359.3	Suppressor Gene
SMARCA4	NM_003072.2	Suppressor Gene
SMARCB1	NM_003073.2	Suppressor Gene
PAK7	NM_020341	Suppressor Gene
SMO	NM_005631.3	Oncogene
APBB1IP	NM_019043.3	Suppressor Gene
SOCS1	NM_003745.1	Suppressor Gene
PRKCA	NM_002737	Suppressor Gene
NEK11	NM_024800.2	Suppressor Gene
TCF7L2	ENST00000369397	Suppressor Gene
STK11	NM_000455	Suppressor Gene
ITK	NM_005546	Suppressor Gene
MAP3K2	NM_006609	Suppressor Gene
ACVR1B	NM_020328	Suppressor Gene
CDC7	NM_003503.2	Suppressor Gene
TGFBR2	NM_003242	Suppressor Gene
SRC	NM_005417	Suppressor Gene
TNFAIP3	NM_006290.2	Suppressor Gene
BMPR1A	NM_004329	Suppressor Gene
ACVR2A	NM_001616	Suppressor Gene
RAD18	NM_020165.2	Suppressor Gene
SUFU	NM_016169.2	Suppressor Gene
TP53	NM_000546	Suppressor Gene
MAP2K7	NM_005043	Suppressor Gene
STK32B	NM_018401	Suppressor Gene
TSHR	NM_000369.1	Oncogene
MGC42105	NM_153361	Suppressor Gene
STK19	NM_032454	Suppressor Gene
UTX	NM_021140.1	Suppressor Gene
VHL	NM_000551.2	Suppressor Gene
LDHB	NM_002300.3	Suppressor Gene
WT1	NM_024426.2	Suppressor Gene
PHOX2B	ENST00000381741	Suppressor Gene

FIG. 5D

Table S2. Characteristics of patients with IPMNs analyzed in this study, including *GNAS* and *KRAS* mutation status.

IPMN #	KRAS mutation	GNAS mutation	Age at surgery	Sex	History of smoking	Post-operative diagnosis	Cyst Grade	Cyst diameter (cm)	Duct type	IPMN Subtype	Cyst location	Sample type
1	G12V	R201C	71	M	No	IPMN	intermediate	3.0	mixed	gastric	body/tail	Cyst wall
2	G12D	R201H	68	M	unknown	IPMN	high	2.0	branch	gastric	body/tail	Cyst wall
3	G12D	R201H	69	M	unknown	IPMN	intermediate	2.0	branch	gastric	head	Cyst wall
4	G12R	R201H	73	F	unknown	IPMN	high	3.2	main	pancreatobiliary	head	Cyst wall
5	G12D & G12R	No mutation detected	66	F	No	IPMN	intermediate	4.0	branch	gastric	entire pancreas	Cyst fluid
6	G12R	R201C	63	M	unknown	IPMN	high	8.0	mixed	intestinal	head	Cyst wall
7	No mutation detected	R201H	64	F	No	IPMN	high	2.0	branch	intestinal	body/tail	Cyst wall
8	G12V	No mutation detected	80	M	No	IPMN	high	2.0	main	gastric	body/tail	Cyst wall
9	G12V & G12D	R201C & R201H	74	M	No	IPMN	high	9.5	main	pancreatobiliary	entire pancreas	Cyst wall
10	G12D	R201C	39	F	No	IPMN	low	2.0	branch	gastric	head	Cyst wall
11	G12D	R201C	67	F	No	IPMN	low	2.0	main	gastric	head	Cyst wall
12	G12V	No mutation detected	70	M	No	IPMN	high	2.0	main	gastric	head	Cyst wall
13	G12V	R201H	78	M	No	IPMN	high	3.0	branch	gastric	head	Cyst wall
14	G12V	R201H	79	M	No	IPMN	high	5.0	branch	gastric	head	Cyst wall
15	G12D	R201C	84	M	No	IPMN	low	1.5	branch	gastric	head	Cyst wall
16	G12D	R201C	66	F	No	IPMN	intermediate	1.8	branch	gastric	head	Cyst wall
17	G12D	R201C	63	F	Yes	IPMN	high	1.8	branch	gastric	body/tail	Cyst wall
18	G12D	R201C	81	F	Yes	IPMN	intermediate	3.0	not determined	gastric	head	Cyst wall
19	G12D	R201H	69	M	Yes	IPMN	low	2.5	not determined	gastric	head	Cyst wall
20	G12V	R201C	70	M	unknown	IPMN	high	5.0	mixed	not determined	body/tail	Cyst wall
21	No mutation detected	R201H	80	M	unknown	IPMN	high	0.7	main	gastric	body/tail	Cyst wall
22	No mutation detected	R201C	60	F	unknown	IPMN	low	1.2	mixed	intestinal	body/tail	Cyst wall
23	No mutation detected	R201C	81	M	unknown	IPMN	high	2.5	main	intestinal	body/tail	Cyst wall
24	G12V	R201C	51	F	unknown	IPMN	intermediate	1.0	mixed	gastric	head	Cyst wall
25	No mutation detected	R201H	78	M	unknown	IPMN	high	3.0	mixed	intestinal	head	Cyst wall
26	G12D	R201H	70	F	unknown	IPMN	intermediate	1.4	branch	not determined	head	Cyst wall
27	G12D	R201C	66	F	unknown	IPMN	low	2.7	mixed	gastric	head	Cyst wall
28	G12D	No mutation detected	62	M	unknown	IPMN	intermediate	3.5	branch	gastric	head	Cyst wall
29	G12D & G12V	No mutation detected	68	M	Yes	IPMN	high	3.5	main	pancreatobiliary	body/tail	Cyst fluid
30	No mutation detected	R201C	65	M	Yes	IPMN	high	6.5	main	gastric	head	Cyst fluid
31	G12D & G12V & G12R	R201H	79	M	unknown	IPMN	high	2.5	branch	not determined	head	Cyst fluid
32	G12R	No mutation detected	74	F	unknown	IPMN	high	5.0	mixed	pancreatobiliary	head	Cyst wall
33	G12V	R201H	82	F	unknown	IPMN	high	0.5	main	not determined	head	Cyst wall
34	G12V	R201C	75	M	No	IPMN	high	2.8	main	gastric	body/tail	Cyst fluid
35	G12V & G12D	R201H	56	F	No	IPMN	low	1.5	branch	gastric	head	Cyst fluid
36	G12D	R201H	69	M	Yes	IPMN	intermediate	2.8	branch	gastric	head	Cyst fluid
37	G12V	No mutation detected	73	M	unknown	IPMN	intermediate	2.0	branch	gastric	body/tail	Cyst fluid
38	No mutation detected	No mutation detected	48	M	No	IPMN	high	2.5	main	not determined	head	Cyst wall
39	G12D	No mutation detected	60	F	Yes	IPMN	low	1.1	mixed	not determined	head	Cyst fluid
40	G12R	R201C	76	F	Yes	IPMN	low	4.0	branch	not determined	tail	Cyst fluid
41	G12D	R201C	72	M	Yes	IPMN	intermediate	1.9	branch	not determined	head	Cyst fluid
42	G12D	R201C	68	M	Yes	IPMN	high	2.0	mixed	not determined	head	Cyst fluid
43	G12D	R201H	76	F	Yes	IPMN	high	0.8	mixed	not determined	body	Cyst fluid
44	G12D	No mutation detected	70	F	No	IPMN	low	5.6	branch	not determined	head	Cyst fluid
45	No mutation detected	R201H	85	F	No	IPMN	intermediate	4.5	mixed	not determined	neck	Cyst fluid
46	No mutation detected	R201H	72	F	No	IPMN	low	2.0	branch	not determined	tail	Cyst fluid
47	G12D	No mutation detected	62	M	No	IPMN	low	2.9	branch	not determined	tail	Cyst fluid
48	G12R	R201C & R201H	84	F	No	IPMN	low	3.2	branch	not determined	tail	Cyst fluid
49	G12D	No mutation detected	71	M	unknown	IPMN	high	1.5	main	not determined	body	Cyst fluid
50	G12D	R201C	70	M	unknown	IPMN	intermediate	3.0	branch	not determined	body	Cyst fluid
51	G12V	No mutation detected	76	F	unknown	IPMN	low	3.4	branch	not determined	body	Cyst fluid

FIG. 6A

52	G12V & G12R	No mutation detected	79	F	unknown	IPMN	intermediate	4.2	mixed	not determined	body	Cyst fluid
53	No mutation detected	No mutation detected	72	F	unknown	IPMN	high	6.7	main	not determined	body/tail	Cyst fluid
54	G12D	R201C	76	F	unknown	IPMN	high	8.5	not determined	not determined	body/tail	Cyst fluid
55	No mutation detected	No mutation detected	81	F	unknown	IPMN	intermediate	1.5	branch	not determined	head	Cyst fluid
56	G12V	R201H	86	M	unknown	IPMN	intermediate	1.5	branch	not determined	head	Cyst fluid
57	G12R	R201C	58	M	unknown	IPMN	intermediate	2.0	branch	not determined	head	Cyst fluid
58	G12D	No mutation detected	66	M	unknown	IPMN	high	2.0	main	not determined	head	Cyst fluid
59	G12V	R201C	81	M	unknown	IPMN	high	2.8	not determined	not determined	head	Cyst fluid
60	G12D & G12V	No mutation detected	68	M	unknown	IPMN	high	2.8	mixed	not determined	head	Cyst fluid
61	G12V	No mutation detected	80	M	unknown	IPMN	high	2.8	main	not determined	head	Cyst fluid
62	G12V	R201H	74	F	unknown	IPMN	intermediate	3.0	branch	not determined	head	Cyst fluid
63	G12V	R201C	65	F	unknown	IPMN	high	3.0	mixed	not determined	head	Cyst fluid
64	G12V	No mutation detected	72	F	unknown	IPMN	low	3.4	branch	not determined	head	Cyst fluid
65	G12D & G12R	R201C	65	F	unknown	IPMN	intermediate	3.5	branch	not determined	head	Cyst fluid
66	G12V	No mutation detected	76	F	unknown	IPMN	intermediate	3.8	branch	not determined	head	Cyst fluid
67	G12V	R201C & R201H	77	F	unknown	IPMN	intermediate	4.0	branch	not determined	head	Cyst fluid
68	No mutation detected	R201C	67	F	unknown	IPMN	intermediate	4.0	mixed	not determined	head	Cyst fluid
69	No mutation detected	R201C	70	M	unknown	IPMN	high	4.0	mixed	not determined	head	Cyst fluid
70	G12V	R201C	52	F	unknown	IPMN	high	4.2	mixed	not determined	head	Cyst fluid
71	G12V	No mutation detected	73	F	unknown	IPMN	intermediate	4.5	branch	not determined	head	Cyst fluid
72	G12D	R201H	74	F	unknown	IPMN	intermediate	5.0	branch	not determined	head	Cyst fluid
73	G12V	R201C	63	M	unknown	IPMN	high	6.0	main	not determined	head	Cyst fluid
74	No mutation detected	R201C	63	M	unknown	IPMN	high	8.5	mixed	not determined	head	Cyst fluid
75	No mutation detected	R201C	67	F	unknown	IPMN	high	7.0	main	not determined	head	Cyst fluid
76	G12D	No mutation detected	79	M	unknown	IPMN	low	13.0	main	not determined	head	Cyst fluid
77	G12V	No mutation detected	86	F	unknown	IPMN	intermediate	1.7	branch	not determined	tail	Cyst fluid
78	G12D & G12V	R201C & R201H	74	M	unknown	IPMN	intermediate	2.0	mixed	not determined	tail	Cyst fluid
79	G12V & G12R	R201H	70	M	unknown	IPMN	intermediate	2.2	branch	not determined	tail	Cyst fluid
80	No mutation detected	R201C	68	M	unknown	IPMN	high	2.5	main	not determined	tail	Cyst fluid
81	G12D	No mutation detected	77	F	unknown	IPMN	high	2.6	mixed	not determined	tail	Cyst fluid
82	G12V	R201C	67	M	unknown	IPMN	intermediate	2.7	branch	not determined	tail	Cyst fluid
83	G12D & G12V	No mutation detected	80	F	unknown	IPMN	intermediate	3.5	mixed	not determined	tail	Cyst fluid
84	G12D	No mutation detected	68	F	unknown	IPMN	intermediate	6.0	branch	not determined	tail	Cyst fluid
85	G12D & G12V	No mutation detected	68	F	unknown	IPMN	intermediate	6.0	branch	not determined	tail	Cyst fluid
86	G12D & G12V	R201C	69	M	unknown	IPMN	high	23.0	main	not determined	tail	Cyst fluid
87	G12D & G12V & G12R	R201C & R201H	69	M	unknown	IPMN	high	23.0	main	not determined	tail	Cyst fluid
88	No mutation detected	R201C	72	M	unknown	IPMN	high	1.4	main	not determined	head	Cyst wall
89	G12D	R201C	74	M	Yes	IPMN	intermediate	3.0	main	gastric	body/tail	Cyst fluid
90	G12V	No mutation detected	54	F	No	IPMN	low	2.0	branch	gastric	body/tail	Cyst fluid
91	No mutation detected	R201H	61	M	No	IPMN	high	1.6	main	intestinal	body/tail	Cyst fluid
92	G12V	R201H	63	F	No	IPMN	intermediate	1.8	branch	not determined	body/tail	Cyst fluid
93	G12D	R201C	73	M	No	IPMN	intermediate	3.2	branch	gastric	body/tail	Cyst fluid
94	G12V	No mutation detected	60	M	No	IPMN	high	4.0	branch	pancreatobiliary	head	Cyst fluid
95	G12D	No mutation detected	75	F	No	IPMN	high	1	branch	gastric	head	Cyst fluid
96	G12D & G12V	R201C	65	M	No	IPMN	intermediate	1.5	branch	gastric	head	Cyst fluid
97	G12V	R201H	67	F	No	IPMN	high	4	branch	gastric	head	Cyst fluid
98	No mutation detected	R201C	54	M	Yes	IPMN	intermediate	4.0	branch	gastric	body/tail	Cyst fluid
99	G12D	No mutation detected	72	M	Yes	IPMN	low	0.5	branch	gastric	body/tail	Cyst fluid
100	G12R	R201H	73	M	Yes	IPMN	intermediate	3.0	branch	gastric	head	Cyst fluid
101	No mutation detected	No mutation detected	79	F	Yes	IPMN	high	4.0	branch	gastric	head	Cyst fluid
102	G12V	No mutation detected	67	F	Yes	IPMN	intermediate	0.6	main	gastric	head	Cyst fluid
103	G12D	No mutation detected	60	F	Yes	IPMN	intermediate	1	branch	not determined	head	Cyst fluid
104	G12V	R201C	54	F	Yes	IPMN	intermediate	1.7	branch	gastric	head	Cyst fluid
105	G12V	R201H	57	M	Yes	IPMN	intermediate	4.5	branch	gastric	head	Cyst fluid
106	No mutation detected	No mutation detected	71	F	unknown	IPMN	low	1.7	branch	gastric	body/tail	Cyst fluid

FIG. 6B

107	G12D	No mutation detected	71	M	unknown	IPMN	low	6.0	branch	not determined	head	Cyst fluid
108	G12D	R201H	66	M	unknown	IPMN	intermediate	0.6	branch	intestinal	head	Cyst fluid
109	No mutation detected	R201H	65	F	unknown	IPMN	intermediate	1.8	mixed	gastric	head	Cyst fluid
110	G12V	R201C	56	M	unknown	IPMN	intermediate	2	main	gastric	head	Cyst fluid
111	G12D	No mutation detected	53	M	unknown	IPMN	intermediate	3.1	branch	gastric	head	Cyst fluid
112	G12R	R201C	62	M	unknown	IPMN	intermediate	3.5	branch	not determined	head	Cyst fluid
113	G12D	R201C	71	M	unknown	IPMN	low	3.5	branch	gastric	head	Cyst fluid
114	G12R	No mutation detected	60	M	No	IPMN	high	13.0	mixed	pancreatobiliary	entire pancreas	Cyst wall
115	G12D	R201H	70	M	No	IPMN	high	4.0	main	pancreatobiliary	head	Cyst wall
116	No mutation detected	R201C	62	F	No	IPMN	high	6.0	main	gastric	head	Cyst wall
117	G12D	R201C	75	M	unknown	IPMN	high	5.0	not determined	gastric	head	Cyst wall
118	G12D	No mutation detected	73	F	Yes	IPMN	high	2.8	branch	gastric	head	Cyst wall
119	G12R	No mutation detected	71	F	No	IPMN	low	4.0	branch	gastric	body/tail	Cyst wall
120	G12V	R201H	72	M	No	IPMN	high	7.0	main	intestinal	body/tail	Cyst wall
121	No mutation detected	R201H	77	M	No	IPMN	high	7.0	main	intestinal	entire pancreas	Cyst wall
122	G12V	R201H	36	M	No	IPMN	high	6.0	main	intestinal	head	Cyst wall
123	G12V	R201H	69	M	No	IPMN	high	5.1	mixed	intestinal	head	Cyst wall
124	G12V	No mutation detected	64	F	Yes	IPMN	low	2.0	branch	gastric	body/tail	Cyst wall
125	No mutation detected	R201C	76	F	Yes	IPMN	high	1.5	main	intestinal	body/tail	Cyst wall
126	G12D	No mutation detected	69	F	unknown	IPMN	intermediate	3.4	mixed	gastric	body/tail	Cyst wall
127	G12D	R201H	79	F	unknown	IPMN	high	14	main	not determined	entire pancreas	Cyst wall
128	G12D	No mutation detected	74	M	unknown	IPMN	intermediate	8.0	main	gastric	head	Cyst wall
129	G12D	No mutation detected	56	M	unknown	IPMN	intermediate	4	branch	gastric	head	Cyst wall
130	G12D & G12V	R201C	68	F	Yes	IPMN	intermediate	2.5	branch	gastric	body/tail	Cyst fluid
131	G12V	R201C	73	F	Yes	IPMN	high	6.0	mixed	intestinal	entire pancreas	Cyst fluid
132	G12V & G12R	R201H	71	M	No	IPMN	intermediate	3.0	mixed	gastric	body/tail	Cyst wall

FIG. 6C

Table S3. Characteristics of patients with cyst types other than IPMN, including *GNAS* and KRAS mutation status.

Cyst #	KRAS	GNAS	Age at surgery	Sex	History of smoking	Post-operative diagnosis	Cyst Grade	Cyst diameter (cm)	Cyst location	Sample type
OT01	No mutation detected	No mutation detected	57	F	unknown	SCA	NA	2.5	head	Cyst fluid
OT02	No mutation detected	No mutation detected	58	F	No	SCA	NA	1.5	Neck	Cyst fluid
OT03	No mutation detected	No mutation detected	54	M	unknown	SCA	NA	2	head	Cyst fluid
OT04	No mutation detected	No mutation detected	57	M	Yes	SCA	NA	2	body/tail	Cyst fluid
OT05	No mutation detected	No mutation detected	69	F	unknown	SCA	NA	3	tail	Cyst fluid
OT06	No mutation detected	No mutation detected	57	F	Yes	SCA	NA	3	body/tail	Cyst fluid
OT07	No mutation detected	No mutation detected	59	F	No	SCA	NA	3	head	Cyst fluid
OT08	No mutation detected	No mutation detected	64	F	No	SCA	NA	3	head	Cyst fluid
OT09	No mutation detected	No mutation detected	60	F	unknown	SCA	NA	3.1	head	Cyst fluid
OT10	No mutation detected	No mutation detected	56	M	Yes	SCA	NA	4	body	Cyst fluid
OT11	No mutation detected	No mutation detected	47	F	Yes	SCA	NA	4	head	Cyst fluid
OT12	No mutation detected	No mutation detected	49	F	unknown	SCA	NA	4	head	Cyst fluid
OT13	No mutation detected	No mutation detected	52	M	unknown	SCA	NA	4.5	tail	Cyst fluid
OT14	No mutation detected	No mutation detected	58	F	unknown	SCA	NA	4.8	tail	Cyst fluid
OT15	No mutation detected	No mutation detected	46	M	Yes	SCA	NA	5	head	Cyst fluid
OT16	No mutation detected	No mutation detected	81	M	unknown	SCA	NA	5	head	Cyst fluid
OT17	No mutation detected	No mutation detected	77	F	unknown	SCA	NA	6	body	Cyst fluid
OT18	No mutation detected	No mutation detected	61	F	unknown	SCA	NA	6	tail	Cyst fluid
OT19	No mutation detected	No mutation detected	72	M	Yes	SCA	NA	7	body/tail	Cyst fluid
OT20	No mutation detected	No mutation detected	61	F	unknown	SCA	NA	7.5	body	Cyst fluid
OT21	No mutation detected	No mutation detected	77	F	Yes	SCA	NA	8	body/tail	Cyst fluid
OT22	No mutation detected	No mutation detected	55	F	unknown	SCA	NA	10	tail	Cyst fluid
OT23	No mutation detected	No mutation detected	61	F	unknown	SCA	NA	13	tail	Cyst fluid
OT24	No mutation detected	No mutation detected	43	M	No	SCA	NA	1.5	head	Cyst fluid
OT25	No mutation detected	No mutation detected	63	M	unknown	SCA	NA	1.8	body/tail	Cyst fluid
OT26	No mutation detected	No mutation detected	62	F	No	SCA	NA	10.5	body/tail	Cyst fluid
OT27	No mutation detected	No mutation detected	57	F	No	SCA	NA	2.5	body/tail	Cyst fluid
OT28	No mutation detected	No mutation detected	86	F	No	SCA	NA	2.5	body/tail	Cyst fluid
OT29	No mutation detected	No mutation detected	58	M	Yes	SCA	NA	2.5	body/tail	Cyst fluid
OT30	No mutation detected	No mutation detected	68	F	unknown	SCA	NA	2.5	head	Cyst fluid
OT31	No mutation detected	No mutation detected	72	F	unknown	SCA	NA	2.8	body/tail	Cyst fluid
OT32	No mutation detected	No mutation detected	63	F	Yes	SCA	NA	3	body/tail	Cyst fluid
OT33	No mutation detected	No mutation detected	47	F	Yes	SCA	NA	3	head	Cyst fluid
OT34	No mutation detected	No mutation detected	64	M	Yes	SCA	NA	3.5	head	Cyst fluid
OT35	No mutation detected	No mutation detected	52	F	No	SCA	NA	3.5	body/tail	Cyst fluid
OT36	No mutation detected	No mutation detected	71	F	No	SCA	NA	3.7	body/tail	Cyst fluid
OT37	No mutation detected	No mutation detected	77	F	Yes	SCA	NA	4	body/tail	Cyst fluid
OT38	No mutation detected	No mutation detected	36	F	unknown	SCA	NA	4.1	head	Cyst fluid

FIG. 7A

OT39	No mutation detected	No mutation detected	74	F	unknown	SCA	NA	4.5	head	Cyst fluid
OT40	No mutation detected	No mutation detected	66	M	No	SCA	NA	5	body/tail	Cyst fluid
OT41	No mutation detected	No mutation detected	40	F	unknown	SCA	NA	5.4	body/tail	Cyst fluid
OT42	No mutation detected	No mutation detected	56	F	No	SCA	NA	5.9	body/tail	Cyst fluid
OT43	No mutation detected	No mutation detected	19	F	unknown	SCA	NA	6.6	head	Cyst fluid
OT44	No mutation detected	No mutation detected	69	M	unknown	SCA	NA	7.5	head	Cyst fluid
OT45	No mutation detected	No mutation detected	32	F	Yes	MCN	Not determined	2.3	body	Cyst fluid
OT46	No mutation detected	No mutation detected	51	F	Yes	MCN	Not determined	2.5	body	Cyst fluid
OT47	G12V	No mutation detected	65	F	No	MCN	Not determined	2.5	tail	Cyst fluid
OT48	G12V	No mutation detected	46	F	No	MCN	low	2.5	tail	Cyst fluid
OT49	G12V	No mutation detected	43	F	Yes	MCN	Not determined	3.2	body	Cyst fluid
OT50	No mutation detected	No mutation detected	46	F	No	MCN	Not determined	3.5	body	Cyst fluid
OT51	No mutation detected	No mutation detected	65	F	Yes	MCN	Not determined	4	tail	Cyst fluid
OT52	No mutation detected	No mutation detected	60	F	Yes	MCN	low	4	body/tail	Cyst fluid
OT53	No mutation detected	No mutation detected	59	F	No	MCN	Not determined	4.5	tail	Cyst fluid
OT54	No mutation detected	No mutation detected	52	F	No	MCN	Not determined	5	body / tail	Cyst fluid
OT55	No mutation detected	No mutation detected	24	F	unknown	MCN	low	5	body/tail	Cyst fluid
OT56	No mutation detected	No mutation detected	43	F	Yes	MCN	Not determined	5.5	body	Cyst fluid
OT57	No mutation detected	No mutation detected	59	F	Yes	MCN	low	7	body/tail	Cyst fluid
OT58	G12V	No mutation detected	42	F	No	MCN	Not determined	8.5	body / tail	Cyst fluid
OT59	No mutation detected	No mutation detected	34	F	unknown	MCN	low	16	head	Cyst fluid
OT60	G12D	No mutation detected	57	F	No	MCN	low	17	body	Cyst fluid
OT61	G12R	No mutation detected	56	F	Yes	MCN	low	1.5	head	Cyst fluid
OT62	G12R	No mutation detected	39	F	unknown	MCN	low	2.5	body/tail	Cyst fluid
OT63	No mutation detected	No mutation detected	36	F	unknown	MCN	low	3.5	head	Cyst fluid
OT64	No mutation detected	No mutation detected	54	F	unknown	MCN	low	5.5	body/tail	Cyst fluid
OT65	No mutation detected	No mutation detected	42	F	unknown	MCN	intermediate	7.4	head	Cyst fluid
OT66	G12D	No mutation detected	45	F	unknown	IOPN	high	3.1	head	Cyst fluid
OT67	No mutation detected	No mutation detected	36	M	unknown	IOPN	high	4.5	head	Cyst fluid
OT68	No mutation detected	No mutation detected	72	M	No	IOPN	high	6.0	head	Cyst fluid
OT69	No mutation detected	No mutation detected	74	M	Yes	IOPN	high	6.0	body/tail	Cyst fluid
OT70	No mutation detected	No mutation detected	55	F	unknown	IOPN	high	10.0	tail	Cyst fluid

FIG. 7B

Fig. 8

Table S4. Quantification of mutations in selected IPMNs containing both *GNAS* and *KRAS* mutations.

IPMN #	*KRAS* mutant allele(s)	Major *KRAS* mutant allele	Major *KRAS* allele freq	Major *KRAS* allele fraction/total mutant alleles*
31	G12D & G12V & G12R	G12D	29%	97%
36	G12D	G12D	18%	Not applicable
41	G12D	G12D	3.0%	NA
42	G12D	G12D	17%	NA
43	G12D	G12D	33%	NA
48	G12R	G12S	1.6%	NA
57	G12R	G12S	0.8%	NA
65	G12D & G12R	G12D	27%	88%
67	G12V	G12V	8.0%	NA
72	G12D	G12D	36%	NA
78	G12D & G12V	G12V	0.9%	65%
79	G12V & G12R	G12V	11%	60%
86	G12D & G12V	G12V	18%	50%
87	G12D & G12V & G12R	G12V	8%	46%
100	G12R	G12S	1.4%	NA
105	G12V	G12V	1.0%	NA
130	G12D & G12V	G12V	4.0%	56%

* NA = Not applicable because there was only a single mutation identified in the IPMN.

Fig. 9

Table S5. Comparison of mutational status in DNA from IPMNs and pancreatic adenocarcinomas from the same patients.

IPMN #	Lesion	*KRAS* mutation	*GNAS* mutation
9	IPMN	G12V & G12D	R201H & R201C
	Cancer	G12V	R201H
11	IPMN	No mutation detected	R201C
	Cancer	No mutation detected	R201C
20	IPMN	G12V	R201C
	Cancer	G12V	R201C
33	IPMN	G12V	R201H
	Cancer	G12R	No mutation detected
122	IPMN	G12V	R201H
	Cancer	G12V	R201H
125	IPMN	No mutation detected	R201C
	Cancer	No mutation detected	R201C
127	IPMN	G12D	R201H
	Cancer	G12D	R201H
131	IPMN	G12V	R201C
	Cancer	G12V	R201C

Fig. 10

Table S6. Oligonucleotide primer and probe sequences.

Gene	Used for:	5'-Modification	Mutation	Sequence (5'-3')*
PCR Amplification Primers				
GNAS	GNAS Forward Primer	None	GNAS 201 R201C, R201H	GGCTTTGGTGAGATCCATTG
GNAS	GNAS Reverse Primer	None	GNAS 201 R201C, R201H	TCCACCTGGAACTTGGTCTC
KRAS	PCR forward primer	None	KRAS G12D, G12R, G12V	GATCATATTCGTCCACAAAATGATTC
KRAS	PCR Reverse Primer	None	KRAS G12D, G12R, G12V	TGACTGAATATAAACTTGTGGTAGTTG
Ligation probes				
GNAS	WT-specific probe	6-FAM	R201H	ATG GAG AAC TTG ACG TCC TG TTC GCT GCC G
GNAS	Mutant-specific probe	HEX	R201H	TTCGCTGCCA
GNAS	Common anchoring probe	Phosphate	R201H	TGT CCT GAC TTC GG TGT CCA CTA GTC ATG CTT
GNAS	WT-specific probe	6-FAM	R201C	ATG GAG AAC TTG ACG TCC AC CTT CGC TGC C
GNAS	Mutant-specific probe	HEX	R201C	CTT *CGC T*G*C T
GNAS	Common anchoring probe	Phosphate	R201C	GTG TCC TGA CTT GG TGT CCA CTA GTC ATG CTT
KRAS	WT-specific probe	6-FAM	G12D	ATG GAG AAC TTG ACG TCC T C CTA CGC CAC
KRAS	Mutant-specific probe	HEX	G12D	TGCCT*ACGC*C*AT
KRAS	Common anchoring probe	Phosphate	G12D	CAG CTC CAA CTA GG TGT CCA CTA GTC ATG CTT
KRAS	WT-specific probe	6-FAM	G12R	TCC CGC GAA ATT AAT ACG AG CTA CGC CACC
KRAS	Mutant-specific probe	HEX	G12R	CTA CGC CAC G
KRAS	Common anchoring probe	Phosphate	G12R	AGC TCC AAC TAC CAC GG TGT CCA CTA GTC ATG CTT
KRAS	WT-specific probe	6-FAM	G12V	ATG GAG AAC TTG ACG TCC T C CTA CGC CAC
KRAS	Mutant-specific probe	HEX	G12V	CCT ACG CCA A
KRAS	Common anchoring probe	Phosphate	G12V	CAG CTC CAA CTA GG TGT CCA CTA GTC ATG CTT
BEAMing probes				
GNAS	Detecting beads containing either WT or mutant sequences	ROX	R201C	CTGAAACAAAATTGAGGT
GNAS	WT-specific probe	Cy3	R201C	AGGACACgGCAGCGA
GNAS	Mutant-specific probe	Cy5	R201C	AGGACACaGCAGCGA
GNAS	Detecting beads containing either WT or mutant sequences	ROX	R201H	CTGAAACAAAATTGAGGT
GNAS	WT-specific probe	Cy3	R201H	CAGGACACGGCAGCG
GNAS	Mutant-specific probe	Cy5	R201H	CAGGACATGGCAGCG
KRAS	Detecting beads containing either WT or mutant sequences	ROX	G12D	TGACGATACAGCTAATTCA
KRAS	WT-specific probe	Cy3	G12D	GGAGCTGGTGGCGTA
KRAS	Mutant-specific probe	Cy5	G12D	GGAGCTGATGGCGTA
KRAS	Detecting beads containing either WT or mutant sequences	ROX	G12V	TGACGATACAGCTAATTCA
KRAS	WT-specific probe	Cy3	G12V	GGAGCTGGTGGCGTA
KRAS	Mutant-specific probe	Cy5	G12V	GGAGCTGTTGGCGTA
KRAS	Detecting beads containing either WT or mutant sequences	ROX	G12R	TGACGATACAGCTAATTCA
KRAS	WT-specific probe	Cy3	G12R	TGGAGCTGGTGGCGT
KRAS	Mutant-specific probe	Cy5	G12R	TGGAGCTCGTGGCGT

* indicates LNA linkages; red font indicates additional nucleotides used to discriminate WT from Mutant sequences in the ligation assays.

ENRICHMENT OF NUCLEIC ACIDS BY COMPLIMENTARY CAPTURE

This invention was made with funds from the United States government. The United States retains certain rights to the invention according to the terms of CA 43460, CA 57345, and CA 62924.

TECHNICAL FIELD OF THE INVENTION

This invention is related to the area of genetic markers. In particular, it relates to methods for enriching nucleic acid sequences for analyses. The nucleic acid sequences may comprise genetic markers, such as cancer or other disease markers.

SUMMARY OF THE INVENTION

According to one aspect of the invention a sample comprising nucleic acids is enriched for target nucleic acid analytes. A set of probes for one or more analytes of interest is synthesized. The probes are complementary to plus or minus strands of a target nucleic acid analyte. Each probe has a common 5' and 3' universal priming site. The set of probes is amplified using primers complementary to the universal priming sites. The amplified probes are ligated to each other to form concatamers. The concatamers are isothermally amplified. The amplified concatamers are bound to a solid support. The solid support is contacted with the sample comprising nucleic acids under hybridization conditions so that complementary nucleic acids in the sample are captured on the solid support and non-complementary nucleic acids are removed. Captured nucleic acids are eluted from the solid support.

According to another aspect of the invention a sample is enriched for target nucleic acid analytes. A set of probes for one or more analytes of interest is synthesized. The probes are complementary to plus or minus strands of a target nucleic acid analyte. Each probe has a common 5' and 3' universal priming site. The set of probes is amplified using primers complementary to the universal priming sites. The amplified probes are ligated together to form concatamers. The concatamers are isothermally amplified in the presence of biotyinylated nucleotides, such that biotinylated nucleotides are incorporated into the concatamers. The amplified concatamers are contacted with the sample nucleic acids to form a mixture. The mixture is subjected to hybridization conditions so that complementary nucleic acids in the mixture hybridize to the concatamers. The mixture is contacted with a solid support which comprises avidin or streptavidin, so that hybridized nucleic acids are captured on the solid support. The solid support is washed to remove nucleic acids which do not comprise biotin. The captured nucleic acids are eluted from the solid support.

These and other embodiments which will be apparent to those of skill in the art upon reading the specification provide the art with methods for assessing, characterizing, and detecting genetic markers, such as cancer markers, and in particular pancreatic cancer markers. In particular, it provides methods for enriching for desired analytes.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** provides a schematic of a capture strategy. Overlapping oligonucleotides flanked by universal sequences complimentary to the 169 genes listed in FIGS. **5**A-D (Table S1) were synthesized on an array. The oligonucleotides were cleaved off the array, amplified by PCR with universal primers, ligated into concatamers and amplified in an isothermal reaction. They were then bound to nitrocellulose filters and used as bait for capturing the desired fragments. An Illumina library was constructed from the sample DNA. The library was denatured and hybridized to the probes immobilized on nitrocellulose. The captured fragments were eluted, PCR amplified and sequenced on an Illumina GAIIX instrument.

FIGS. **2**A-**2**B show a ligation assays used to assess KRAS (v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog) and GNAS (guanine nucleotide binding protein (G protein), alpha stimulating activity polypeptide 1) mutations. (FIG. **2**A) Schematic of the ligation assay. Oligonucleotide probes complementary to either the WT or mutant sequences were incubated with a PCR product containing the sequence of interest. The WT- and mutant-specific probes were labeled with the fluorescent dyes 6-FAM and HEX, respectively, and the WT-specific probe was 11 bases longer than the mutant-specific probe. After ligation to a common anchoring primer, the ligation products were separated on a denaturing polyacrylamide slab gel. Further details of the assay are provided in the Materials and Methods. (FIG. **2**B) Examples of the results obtained with the ligation assay in the indicated patients. Templates were derived from DNA of normal duodenum or IPMN tissue. Each lane represents the results of ligation of one of four independent PCR products, each containing 200 template molecules. The probe in the left panel was specific to the GNAS R201H mutation and the probe on the right panel was specific for the GNAS R201C mutation.

FIG. **3** shows BEAMing assays used to quantify mutant representation. PCR was used to amplify KRAS or GNAS sequences containing the region of interest (KRAS codon 12 and GNAS codon 201). The PCR-products were then used as templates for BEAMing, in which each template was converted to a bead containing thousands of identical copies of the templates (34). After hybridization to Cy3- or Cy5-labeled oligonucleotide probes specific for the indicated WT or mutant sequences, respectively, the beads were analyzed by flow cytometry. Scatter plots are shown for templates derived from the DNA of IPMN 130 or from normal spleen. Beads containing the WT or mutant sequences are widely separated in the scatter plots, and the fraction of mutant-containing beads are indicated. Beads whose fluorescence spectra lie between the WT and mutant-containing beads result from inclusion of both WT and mutant templates in the aqueous nanocompartments of the emulsion PCR.

FIGS. **4**A-**4**C show IPMN morphologies. (FIG. **4**A) H&E-stained section of a formalin-fixed, paraffin embedded sample (shows two apparently independent IPMNs with distinct morphologies located close to one another. The IPMN of gastric epithelial subtype (black arrow) harbored a GNAS R201C and a KRAS G12'V while the IPMN showing the intestinal subtype (red arrow) contained a GNAS R201C mutation but no KRAS mutation. (FIG. **4**B) H&E stained section of a different, typical IPMN (FIG. **4**C) Same IPMN as in FIG. **4**B after microdissection of the cyst wall.

FIGS. **5**A-**5**D. (Table S1.) Genes analyzed by massively parallel sequencing in IPMN cyst fluids.

FIGS. **6**A-**6**C. (Table S2.) Characteristics of patients with IPMNs analyzed in this study, including GNAS and KRAS mutation status.

FIGS. **7**A-**7**B. (Table S3.) Characteristics of patients with cyst types other than IPMN, including GNAS and KRAS mutation status.

FIG. **8**. (Table S4.) Quantification of mutations in selected IPMNs containing both GNAS and KRAS mutations.

FIG. **9**. (Table S5.) Comparison of mutational status in DNA from IPMNs and pancreatic adenocarcinomas from the same patients.

FIG. **10**. (Table S6.) Oligonucleotide primer and probe sequences (SEQ ID NO: 4-38).

DETAILED DESCRIPTION OF THE INVENTION

Capturing and amplifying analyte nucleic acids from dilute samples can be particularly taxing. Enrichment for desired sequences can make assays feasible that would otherwise fall below detection limits. Test samples can be from any appropriate source in the patient's body that will have nucleic acids from a cancer or lesion that can be collected and tested. In some cases the nucleic acids will be amplified prior to testing. Suitable test samples may be obtained from pancreatic cyst fluid, pancreatic cyst wall tissue, pancreatic ductal adenocarcinoma tissue, blood, stool, saliva, sputum, bronchoalveolar lavage, urine, and pancreatic juice. The samples may be collected using any means conventional in the art, including from surgical samples, from biopsy samples, from endoscopic ultrasound (EUS), phlebotomy, etc. Obtaining the samples may be performed by the same person or a different person that conducts the subsequent analysis. Samples may be stored and/or transferred after collection and before analysis. Samples may be fractionated, treated, purified, enriched, prior to assay.

Solid supports which may be used are any which are convenient for the particular purpose and situation. These may be filters, beads, magnetic beads, plastic surfaces, microtiter plates, resins, etc. The supports can be treated and derivatized as is known in the art. In particular assays, attachment of a specific binding pair member may be used. For example, avidin or streptavidin may be used as one binding pair member, and biotin as another. One binding pair member may be used on the nucleic acid analyte and one binding pair member may be on the solid support. As exemplified below, biotin can be incorporated into a nucleic acid analyte using biotinylated dNTPs during amplification or synthesis. Other binding pairs which provide a strong bond may be used as well.

Isothermal amplification is one means for producing large amounts of particular sequences. Isothermal amplification is also known as Multiple Displacement Amplification or rolling circle amplification. Other means as are known can also be used.

Ligation reactions are used to join together individual oligonucleotide probes into long polymers or concatamers. Ligation reaction conditions and enzymes for performing these reactions are known in the art and can be used as is convenient.

Any means of testing for a mutation, including without limitation, a point mutation, a deletion, an amplification, a loss of heterozygosity, a rearrangement, a duplication, may be used. As an example, a mutation in codon 201 of GNAS or codon 12 of KRAS may be assayed. Any means of testing for a mutation may be used. Mutations may be detected by sequencing, by hybridization assay, by ligation assay, etc. If locations of the relevant mutations are defined, specific assays which focus on the identified locations may be used. Identifying a mutation as somatic can be accomplished by comparing a test sample to a non-neoplastic sample, either from the same patient or from a healthy individual. The defined locations of some mutations permit focused assays limited to an exon, domain, or codon. But non-targeted assays may also be used, where the location of a mutation is unknown. Any assay that is performed on a test sample involves a transformation, for example, a chemical or physical change or act. Assays and determinations are not performed merely by a perceptual or cognitive process in the body of a person.

Probes and/or primers may contain the wild-type or a mutant sequence. These can be used in a variety of different assays, as will be convenient for the particular situation. Selection of assays may be based on cost, facilities, equipment, electricity availability, speed, reproducibility, compatibility with other assays, invasiveness of sample collection, sample preparation, etc.

Any of the assay results may be recorded or communicated, as a positive act or step. Communication of an assay result, diagnosis, identification, or prognosis, may be, for example, orally between two people, in writing, whether on paper or digital media, by audio recording, into a medical chart or record, to a second health professional, or to a patient. The results and/or conclusions and/or recommendations based on the results may be in a natural language or in a machine or other code. Typically such records are kept in a confidential manner to protect the private information of the patient.

Collections of probes, primers, control samples, and reagents can be assembled into a kit for use in the methods. The reagents can be packaged with instructions, or directions to an address or phone number from which to obtain instructions. An electronic storage medium may be included in the kit, whether for instructional purposes or for recordation of results, or as means for controlling assays and data collection.

Control samples can be obtained from the same patient from a tissue that is not apparently diseased. Alternatively, control samples can be obtained from a healthy individual or a population of apparently healthy individuals. Control samples may be from the same type of tissue or from a different type of tissue than the test sample.

The data described below document the existence of a heretofore unappreciated molecular pathway leading to pancreatic neoplasia. There is no doubt that GNAS mutations plays a driving role in this IPMN-specific pathway: the mutations are remarkably common and they occur at a single codon (201), mutations of which are known to endow cells with extremely high adenyl cyclase activity and cAMP levels (37-39). Based on their rate of mutation and specificity (30), the probability that these mutations are passengers rather than drivers of IPMN development is negligible.

The data also demonstrated that >96% of IPMNs have either a GNAS or KRAS (v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog) mutation and more than half have both mutations. Which mutation—KRAS or GNAS—arises first? There were 20 cases in which GNAS mutations were identified in the absence of KRAS mutations and six additional cases in which GNAS mutations were at least 5 times more abundant than KRAS mutations in the same cyst fluid (FIG. **8** (Table S4)). The converse situation—KRAS mutations in the absence of GNAS mutations—was also observed in many cases (FIG. **6** (Table S2)). These data, in combination with the demonstration that more than one KRAS or more than one GNAS mutation could be identified in the same cyst (FIG. **8** (Table S4)), suggests two models for IPMN development. First, it is possible that IPMN locules represent independent entities whose evolution is unrelated to other locules within the same IPMN. This model is

inconsistent with our data because two adjacent locules within a single grossly distinct IPMN were more likely to contain the same KRAS or GNAS mutation than the lining epithelium from two topographically different cysts, as noted in the Results section. Second, it is possible that all IPMNs are initiated by a single founding mutation in either GNAS or in KRAS. Subsequent mutations of cells within the cystic lesion would lead to independent clonal expansions, perhaps represented by different locules. Such polyclonality has been observed in colorectal adenomas, which are initiated by mutations in APC pathway genes but sometimes progress through heterogeneous KRAS mutations to a transient polyclonal stage (40). This stage is eventually replaced by subsequent clonal expansion of a cell with one of these KRAS mutations (40). A related possibility is that IPMNs are initiated by a genetic or epigenetic alteration in a gene other than KRAS or GNAS, and that we have observed subsequent clonal expansions of these initiated cells. Finally, it is possible that most IPMNs are indeed initiated by a mutation (in GNAS, KRAS, or another gene), but that occasionally two such IPMNs, initiated by completely different cells, develop adjacent to one another. This appeared to be the situation for the case shown in FIG. **4**A, for example. Though these models are difficult to distinguish from one another, it is possible that lineage tracking can be accomplished by complete sequencing of IPMN locule genomes in the future (41).

Apart from its implications for understanding IPMN development, our data have potentially important practical ramifications. The appropriate management of a patient with a pancreatic cyst depends on the type of cyst (42). In particular, it is generally agreed that there is no need to remove asymptomatic SCAs because these lesions have a vanishingly small malignant potential (43). However, the distinction between SCA and mucinous cystic lesions (IPMN and MCN) of the pancreas is often not easy, even after extensive imaging and follow-up (6). One example of these difficulties is provided by the nature of the lesions in our study: the great majority of the 44 SCAs we examined were removed because they were preoperatively believed to be cysts with malignant potential. Hence, many of these 44 surgical procedures were likely unnecessary.

These diagnostic difficulties have long been appreciated and have spurred attempts to develop biomarkers as adjuncts to clinical data, imaging, and cytology (44). Indeed, new protein and glycoprotein markers are showing promising results (45, 46). One conceptual disadvantage of these protein biomarkers is that they are simply associated with cyst development and do not play a pathogenic role. Alterations of oncogenes such as KRAS are attractive alternatives because they are intimately involved in pathogenesis (47-50). In the largest previous study to date on such alterations, 45% of the fluids from mucinous cysts were shown to contain KRAS mutations (25). Our data demonstrates that KRAS mutations are actually present in a larger fraction of IPMNs, probably a result of the more sensitive methods used in our study combined with optimization of procedures used to purify cyst fluid DNA (see Materials and Methods). Third, and most important, the combination of GNAS and KRAS mutation detection provides high sensitivity and specificity for distinguishing between SCAs and IPMNs. The vast majority of IPMNs had a GNAS and/or a KRAS (95% CI 91% to 99%) while no SCAs had either mutation. These data indicate a sensitivity of 0.96 (95% CI 0.91 to 0.99) and a specificity of 1.0 (97.5% one-sided CI 0.92 to 1) for distinguishing between these two lesions. In addition, although not as sensitive, the presence of a GNAS mutation in cyst fluid can also distinguish IPMNs from MCNs (FIG. **7** (Table S3)). The assay involves just two amplicons (GNAS and KRAS) and can be performed with as little as 250 ul of cyst fluid.

Several caveats to the potential utility of such tests should be noted. First, the analysis of cyst fluid obtained through EUS, though safe, is an invasive procedure. Complications include bleeding, infection, and pancreatitis, are reversible, and are generally observed in <1% of patients (reviewed in (51)). Second, neither KRAS nor GNAS mutations can distinguish high grade or invasive from low grade IPMNs. The supplementation of KRAS and GNAS mutational analyses with other markers indicative of grade would clearly be useful (11). Third, we cannot yet reliably distinguish IPMNs from MCNs through the analysis of cyst fluid. Although MCNs do not contain GNAS mutations, a third of them contain KRAS mutations (FIG. **7** (Table S3)) MCN-specific mutations may be identified in the future through a strategy similar to the one we used to identify mutations in IPMNs.

Astute clinicians and pathologists have long suspected that adenocarcinomas of the pancreas arising in IPMNs are a "different disease" than those arising locally distant or in the absence of an IPMN (15, 52). We here provide evidence in support of this hypothesis and identify a key molecular component that underlies this difference.

The above disclosure generally describes the present invention. All references disclosed herein are expressly incorporated by reference. A more complete understanding can be obtained by reference to the following specific examples which are provided herein for purposes of illustration only, and are not intended to limit the scope of the invention.

EXAMPLE 1

Materials and Methods

Patients and Specimens

The present study was approved by the Institutional Review Boards of Johns Hopkins Medical Institutions, Memorial Sloan Kettering Cancer Center and the University of Indiana. We included individuals in whom pancreatic cyst fluid samples from pancreatectomy specimens and/or fresh frozen tumor tissues were available for molecular analysis. Relevant demographic, clinicopathologic data were obtained from prospectively maintained clinical databases and correlated with mutational status.

Pancreatic cyst fluids were harvested in the Surgical Pathology suite from surgically resected pancreatectomy specimens with a sterile syringe. Aspirated fluids were stored at −80° C. within 30 min of resection. Fresh-frozen tissue specimens of surgically resected cystic neoplasms of the pancreas were obtained through a prospectively maintained Johns Hopkins Surgical Pathology Tumor Bank. These lesions as well as normal tissues were macrodissected using serial frozen sections to guide the trimming of OCT embedded tissue blocks to obtain a minimum neoplastic cellularity of 80%. Formalin-fixed and paraffin-embedded archival tissues from surgically resected pancreata were sectioned at 6 μm, stained with hematoxylin and eosin, and dissected with a sterile needle on a SMZ1500 stereomicroscope (Nikon). An estimated 5,000-10,000 cells were microdissected from each lesion. Lesions were classified as IPMNs, MCNs, or SCAs using standard criteria (53). IPMNs were subtyped by internationally accepted criteria (54).

DNA Purification

DNA was purified from frozen cyst walls using an AllPrep kit (Qiagen) and from formalin-fixed, paraffin-embedded sections using the QIAamp DNA FFPE tissue kit (Qiagen) according to the manufacturer's instructions. DNA was purified from 250 μL of cyst fluid by adding 3 ml RLTM buffer (Qiagen) and then binding to an AllPrep DNA column (Qiagen) following the manufacturer's protocol. DNA was quantified in all cases with qPCR, employing primers and conditions as described (55).

Illumina Library Preparation

Cyst fluid DNA was first quantified through real-time PCR using primers specific for repeated sequences in DNA (LINE) as described (56). A minimum of 100 ng DNA from cyst fluid was used to make Illumina libraries according to manufacturer's protocol with the exception that the amount of adapters was decreased in proportional fashion when a lower amount of template DNA was used. The number of PCR cycles used to amplify the library after ligation of adapters was varied to ensure a yield of ~5 ug of the final library product for capture.

Target DNA Enrichment

The targeted region included all of the 3386 exons of 169 cancer related genes and was enriched with custom-made oligonucleotide probes. The design of each oligonucleotide was as follows: 5'-TCCCGCGACGAC-36 bases from the genomic region of interest -GCTGGAGTCGCG-3' (SEQ ID NO: 1). Probes were designed to capture both the plus and the minus strand of the DNA and had a 33-base overlap. The probes were custom-synthesized on a chip. The oligonucleotides were cleaved from the chip by treatment for five hours with 3 ml 35% ammonium hydroxide at room temperate. The solution was transferred to two 2-ml tubes, dried under vacuum, and re-dissolved in 400 ul RNase and DNase free water. Five ul of the solution were used for PCR amplification with primers complementary to the 12 base sequence common to all probes: 5-TGATCCCGCGACGA*C-3' (SEQ ID NO: 2), 5'-GACCGCGACTCCAG*C-3' (SEQ ID NO: 3), with * indicating a phosphorothioate bond. The PCR mix contained 27 ul H_2O, 5 ul template DNA, 2 ul forward primer (25 uM), 2 ul reverse primer (25 uM), 4 ul $MgCl_2$ (50 mM), 5 ul 10× Platinum Taq buffer (Life Technologies), 4 ul dNTPs (10 mM each) and 1 ul Platinum Taq (5 U/ul, Life Technologies). The cycling conditions were: one cycle of 98° C. for 30 s; 35 cycles of 98° C. for 30 s, 40° C. for 30 s, 60° C. for 15 s, 72° C. for 45 s; one cycle of 72° C. for 5 min. The PCR product was purified using a MinElute Purification Column (Qiagen) and end-repaired using End-IT DNA End-Repair Kit (Epicentre) as follows: 34 ul DNA, 5 ul 10× End-Repair Buffer, 5 ul dNTP Mix, 5 ul ATP, 1 ul End-Repair Enzyme Mix. The mix was incubated at room temperature for 45 minutes, and then purified using a MinElute Purification Column (Qiagen). The PCR products were ligated to form concatamers using the following protocol: 35 ul End-Repaired DNA product, 40 ul 2×T4 DNA ligase buffer, 5 ul T4 DNA ligase (3000 units; Enzymatics Inc.) The mix was incubated at room temperature for 4 hours, then purified using QiaQuick Purification Column (Qiagen), and quantified by absorption at 260 nm.

Replicates of 50 ng of concatenated PCR product were amplified in 25 ul solution using the REPLI-g midi whole genome amplification kit (Qiagen) according to the manufacturer's protocol. The RepliG-amplified DNA (20 ug) was then bound to a nitrocellulose membrane and used to capture DNA libraries as described (57). In general, 5 ug of library DNA were used per capture. After washing, the captured libraries were ethanol precipitated and redissolved in 20 ul TE buffer. The DNA was then amplified in a PCR mix containing 51 ul dH_2O, 20 ul 5× Phusion buffer, 5 ul DMSO, 2 ul 10 mM dNTPs, 50 pmol Illumina forward and reverse primers, and 1 ul Hotstart Phusion enzyme (New England Biolabs) using the following cycling program: 98° C. for 30 sec; 15 cycles of 98° C. for 25 sec., 65° C. for 30 sec, 72° C. for 30 sec; and 72° C. for 5 min. The amplified PCR product was purified using a NucleoSpin column (Macherey Nagel, inc.) according to the manufacturer's suggested protocol except that the NT buffer was not diluted and the DNA bound to the column was eluted in 35 ul elution buffer. The captured library was quantified with realtime PCR with the primers used for grafting to the Illumina sequencing chip.

Ligation Assay

PCR products containing codon 12 of KRAS and codon 201 of GNAS were amplified using the primers described in FIG. **10** (Table S6). Each 10-ul PCR contained 200 template molecules in 5 ul of 2× Phusion Flash PCR Master Mix (New England Biolabs) and final concentrations of 0.25 uM forward and 1.5 uM reverse primers. Note that the mutant-specific probes sometimes included locked nucleic acid residues (FIG. **10** (Table S6); Exiqon). The following cycling conditions were used: 98° C. for 2 min; 3 cycles of 98° C. for 10 sec., 69° C. for 15 sec, 72° C. for 15 sec; 3 cycles of 98° C. for 10 sec., 66° C. for 15 sec, 72° C. for 15 sec; 3 cycles of 98° C. for 10 sec., 63° C. for 15 sec, 72° C. for 15 sec; 41 cycles of 98° C. for 10 sec., 60° C. for 60 sec. Reactions were performed in at least quadruplicate and each was evaluated independently. Five ul of a solution containing 0.5 ul of Proteinase K, (18.8 mg/ml, Roche) and 4.5 ul of dH_2O was added to each well and incubated at 60° C. for 30 minutes to inactivate the Phusion polymerase and then for 10 min at 98° C. to inactivate the Proteinase K.

The ligation assay was based on techniques described previously, using thermotolerant DNA ligases (58-61). Each 10-ul reaction contained 2-ul of PCR product (unpurified), 1 ul of 10× Ampligase buffer (Epicentre), 0.5 ul of Ampligase (5 U/ul, Epicentre), anchoring primer (final concentration 2 uM), WT-specific primer (final concentration 0.1 uM), and mutant-specific primer (final concentration 0.025 uM). The sequences of these primers are listed in FIG. **10** (Table S6). The following cycling conditions were used: 95° C. for 3 min; 35 cycles of 95° C. for 10 sec., 37° C. for 30 sec, 45° C. for 60 sec. Five ul of each reaction was added to 5 ul of formamide and the ligation products separated on a 10% Urea-Tris-Borate-EDTA gel (Invitrogen) and imaged with an Amersham-GE Typhoon instrument (GE Healthcare).

BEAMing Assays

These were performed as described (62) using the PCR products generated for the ligation assay as templates and the oligonucleotides listed in FIG. **10** (Table S6) as hybridization probes.

Statistical Analysis

Fisher's exact tests were used to compare the differences between proportions and Wilcoxon Rank Sum tests were used to compare differences in mutational status by age. Confidence intervals for the prevalence of mutations were estimated using the binomial distribution. To compare the prevalence of mutations in grossly distinct IPMNs to adjacent locules within a single grossly distinct IPMN, we compared the probability of observing given KRAS or GNAS mutation in the 111 distinct IPMNs to conditional probability that given the first locule sequenced contained a specific KRAS or GNAS mutation all other locules contained the same KRAS or GNAS mutations. The probabilities of GNAS or KRAS mutations occurring by chance was calculated using a binomial distribution and the previously

estimated mutation rates of tumors or normal cells (30). STATA version 11 was used for all statistical analysis (63).

EXAMPLE 2

Massively Parallel Sequencing of 169 Genes in Cyst Fluid DNA

To initiate this study, we determined the sequences of 169 presumptive cancer genes in the cyst fluids of 19 IPMNs, each obtained from a different patient. Thirty-three of the 169 were oncogenes and the remainder were tumor suppressor genes. Though only a tiny subset of these 169 genes were known to be mutated in PDAs, all were known to be frequently mutated in at least one solid tumor type (FIG. **5** (Table S1)). We additionally sequenced these genes in normal pancreatic, splenic or intestinal tissues of the same patients to determine which of the alterations identified were somatic. We chose to use massively parallel sequencing rather than Sanger sequencing for this analysis because we did not know what fraction of DNA purified from the cyst fluid was derived from neoplastic cells. Massively parallel sequencing has the capacity to identify mutations present in 2% or more of the studied cells while Sanger sequencing often requires >25% neoplastic cells for this purpose. IPMNs are by definition connected with the pancreatic duct system and the cyst fluid containing cellular debris and shed DNA from the neoplastic cells can be expected to be admixed with that of the cells and secretions derived from normal ductal epithelial cells.

We devised a strategy to capture sequences of the 169 genes from cyst fluid DNA (FIG. **1**). In brief, 244,000 oligonucleotides, each 60 bp in length and in aggregate covering the exonic sequences of all 169 genes, were synthesized in parallel using phosphoramadite chemistry on a single chip synthesized by Agilent Technologies. After removal from the chip, the oligonucleotide sequences were amplified by PCR and ligated together. Multiple displacement amplification was then used to further amplify the oligonucleotides, which were then bound to a filter. Finally, the filter was used to capture complementary DNA sequences from the cyst fluids and corresponding normal samples, and the captured DNA was subjected to massively parallel sequencing.

The target region corresponding to the coding exons of the 169 genes encompassed 584,871 bp. These bases were redundantly sequenced, with 902±411 (mean±1 SD) fold-coverage in the 38 samples sequenced (19 IPMN cyst fluids plus 19 matched DNA samples from normal tissues of the same patients). This coverage allowed us to confidently detect somatic mutations present in >5% of the template molecules.

There were only two genes mutated in more than one IPMN—KRAS, which was mutated in 14 of the 19 IPMNs, and GNAS, which was mutated in 6 IPMNs. The mutations in GNAS all occurred at codon 201, resulting in either a R201H or R201C substitution. GNAS is a well-known oncogene that is mutated in pituitary and other uncommon tumor types (16-19). However, such mutations have rarely been reported in common epithelial tumors (20-22). In pituitary tumors, mutations cluster at two positions—codons 201 and 227 (16, 19). This clustering provides extraordinary opportunities for diagnosis, similar to that of KRAS. For example, the clustering of KRAS mutations has facilitated the design of assays to detect mutations in tumors of colorectal cancer patients eligible for therapy with antibodies to EGFR (23). All twelve KRAS mutations identified through massively parallel sequencing of cyst fluids were at codon 12, resulting in a G12D, G12V, or G12R amino acid change. KRAS mutations at codon 12 have previously been identified in the vast majority of PDAs as well as in 40 to 60% of IPMNs (24-29). GNAS mutations have not previously been identified in pancreatic cysts or in PDAs.

EXAMPLE 3

Frequency of KRAS and GNAS Mutations in Pancreatic Cyst Fluid DNA

We next determined the frequency of KRAS codon 12 and GNAS codon 201 mutations in a larger set of IPMNs. The clinical characteristics of all IPMNs analyzed in this study are listed in FIG. **6** (Table S2). To ensure that the analyses were performed robustly, we carried out preliminary experiments with cyst fluids from patients with known mutations based on the massively parallel sequencing experiments described above. We tested several methods for purifying DNA from often viscous cyst fluids and used the optimum method for subsequent experiments. Quantitative PCR was used to determine the number of amplifiable template molecules recovered with this procedure. In eight cases, we compared pelleted cells to supernatants derived from the same cyst fluid samples and found that the fraction of mutant templates in both compartments was similar. On the basis of these results, we purified DNA from 0.25 ml of whole cyst fluid (cells plus supernatant) and, as assessed by quantitative PCR, recovered an average of 670±790 ng of usable DNA.

For each of 84 cyst fluid samples (an independent cohort of 65 patients plus the 19 patients whose fluids had been studied by massively parallel sequencing), we analyzed ~800 template molecules for five distinct mutations, three at KRAS codon 12 and two within GNAS codon 201 (see Materials and Methods). A PCR/ligation method that had the capacity to detect one mutant template molecule among 200 normal (wild-type, WT) templates was used for these analyses (FIG. **2**A). We identified GNAS and KRAS mutations in 61% and 82% of the IPMN fluids, respectively (representative examples in FIG. **2**B). In those samples without GNAS codon 201 mutations, we searched for GNAS codon 227 mutations, but did not find any. We also analyzed macro- and microdissected frozen or paraffin-embedded cyst walls from an independent collection of 48 surgically resected IPMNs, and similarly identified a high prevalence of GNAS (75%) and KRAS (79%) mutations. In aggregate, 66% of 132 IPMNs harbored a GNAS mutation, 81% harbored a KRAS mutation, slightly more than half (51%) harbored both GNAS and KRAS mutations, while at least one of the two genes was mutated in 96.2% (FIG. **6** (Table S2)). Given background mutation rates in tumors or normal tissues (30), the probability that either GNAS or KRAS mutations occurred by chance alone was less than 10^{-30}. There were no significant correlations between the prevalence of KRAS or GNAS mutations and age, sex, or smoking history of the patients ($P>0.05$) (Table 1). Small (<3 cm) as well as larger cysts had similar fractions of both KRAS and GNAS mutations and the location of the IPMN (head, body, or tail) did not correlate with the presence of mutation in either gene (Table 1). GNAS and KRAS mutations were present in low-grade as well as in high-grade IPMNs. The prevalence of KRAS mutations was higher in lower grade lesions ($P=0.03$) whereas the prevalence of GNAS mutations was somewhat higher in more advanced lesions ($P=0.11$) (Table 1). GNAS, as well as KRAS mutations were present in each of the three major histologic types of IPMNs—intestinal, pancreatobiliary, and gastric. However, the prevalence of the mutations varied across the histological types ($P<0.01$ for

both KRAS and GNAS). GNAS mutations were most prevalent in the intestinal subtype (100%), KRAS mutations had the highest frequency (100%) in the pancreatobiliary subtype and had the lowest frequency (42%) in the intestinal subtype (Table 1).

We then determined whether GNAS mutations were present in SCAs, a common but benign type of pancreatic cystic neoplasm. We examined a total of 44 surgically resected SCAs, each from a different patient (42 cyst fluids and 2 cyst walls). Many of these cysts were surgically resected because they clinically mimicked an IPMN. They would have likely not been surgically excised had they been known to be SCAs. The SCAs averaged 5.0±2.8 cm in maximum diameter (FIG. **7** (Table S3)) similar to the IPMNs (4.4±3.7 maximum diameter, FIG. **6** (Table S2)). There was little difference in the locations of the SCAs and IPMNs within the pancreas (FIGS. **6** and **7** (Tables S2 and S3)). However, no GNAS or KRAS mutations were identified in the SCAs, in marked contrast to the IPMNs ($p<0.001$, Fisher's Exact Test). GNAS mutations were also not identified in any of 21 MCNs ($p=0.005$ when compared to IPMNs, Fisher's Exact Test), although KRAS mutations were found in 33% of MCNs (FIG. **7** (Table S3)). GNAS mutations were also not identified in five examples of an uncommon type of cyst, called intraductal oncocytic papillary neoplasm (IOPN), with characteristic oncocytic features (FIG. **7** (Table S3)).

We investigated clonality in more detail by precisely quantifying the levels of mutations in the subset of cyst fluids containing more than one mutation of the same gene. To accomplish this, we used a technique called BEAMing (34). Through this method, individual template molecules are converted into individual magnetic beads attached to thousands of molecules with the identical sequence. The beads are then hybridized with mutation-specific probes and analyzed by flow cytometry (FIG. **3**). The analysis of 17 IPMN cyst fluids, each with mutations in both KRAS and GNAS, showed that the fraction of mutant alleles varied widely, ranging from 0.8% to 45% of the templates analyzed. There was an average of 12.8%±12.2% mutant alleles of KRAS and an average of 24.4±13.1% mutant alleles of GNAS in the 17 IPMN cyst fluids examined (FIG. **8** (Table S4)). In two of the seven IPMNs with more than one KRAS mutation, there was a predominant mutant that out-numbered the second KRAS mutant by >5:1 (FIG. **8** (Table S4)). Similarly, two of the four cases harboring two different GNAS mutations had a predominant mutant (FIG. **8** (Table S4)). In the other cases, the different mutations in KRAS (or GNAS) were distributed more evenly (FIG. **8** (Table S4)). These data support the idea that cells within a subset of IPMNs had undergone independent clonal expansions, giving rise to apparent polyclonality (35).

TABLE 1

Correlations between mutations and clinical and histopathologic parameters of IPMNs

		N,	KRAS mutation		P-	GNAS mutation		P-
		total	N	%	value	N	%	value
Age in years	<65 years	29	22	75.9	0.42	18	62.1	0.62
	≥65 years	103	85	82.5		69	67	
Gender	Male	70	58	82.9	0.58	51	72.9	0.07
	Female	62	49	79		36	58.1	
History of smoking	Yes	25	21	84	0.77	17	68	0.85
	No	37	30	81.1		26	70.3	
Grade	Low	23	20	87	0.43	11	47.8	0.04
	Intermediate	51	46	90.2	(low vs.	34	66.7	(low vs.
	High	58	41	70.7	others)	42	72.4	others)
Duct type	Main	35	23	65.7	0.002	24	68.6	0.37
	Branch	64	58	90.6	(main vs.	38	59.4	(main vs.
	Mixed	28	21	75	branch)	20	71.4	branch)
Subtype	Gastric	52	45	86.5	0.02	34	65.4	0.002
	Pancreatobiliary	7	7	100	(panc. vs	3	42.9	(panc. vs
	Intestinal	13	6	46.2	intestinal)	13	100	intestinal)
Diameter	<3 cm	62	49	79	0.58	41	66.1	0.96
	≥3 cm	70	58	82.9		46	65.7	
Location	Proximal (head)	77	64	83.1	0.44	53	68.8	0.38
	Distal (body, tail)	49	38	77.6	(prox. vs	30	61.2	(prox. vs
	Proximal and distal	6	5	83.3	distal)	4	66.7	distal)
Associated cancer	Yes	24	18	75	0.4	18	75	0.3
	No	108	89	82.4		69	63.9	

EXAMPLE 4

IPMN Polyclonality

KRAS G12D, G12V, and G12R mutations were found in 43%, 39%, and 13% of IPMNs, respectively (FIG. **6** (Table S2)). A small fraction (11%) of the IPMNs contained two different KRAS mutations and 2% contained three different mutations. Likewise, GNAS R201C and GNAS R201H mutations were present in 39% and 32% of the IPMNs, respectively, and 4% of the IPMNs had both mutations (FIG. **6** (Table S2)). More than one mutation in KRAS in IPMNs has been observed in prior studies of IPMNs (31-33) and the multiple KRAS and GNAS mutations are suggestive of a polyclonal origin of the tumor.

IPMNs are often multilocular or multifocal in nature, looking much like a bunch of grapes (FIG. **4**A) (36). To determine the relationship between cyst locules (individual grapes) and cyst fluid, we microdissected the walls from individual locules of each of ten IPMNs from whom cyst fluid was available (example in FIGS. **4**B and C). The individual locule walls generally appeared to be monoclonal, as more than one KRAS mutation was only found in one (4.5%) of the 22 locules examined. No locule wall contained more than one GNAS mutation and two adjacent locules within a single grossly distinct IPMN were more likely to contain the same KRAS or GNAS mutation than the lining epithelium from two topographically different IPMNs ($p<0.05$, Fisher's Exact Test for KRAS G12D, KRAS G12R

and GNAS R201H mutations; P<0.10 for KRAS G12V and GNAS R201H mutations). All of the ten KRAS and six GNAS mutations identified in the cyst fluid could be identified in the corresponding locule walls. These data leave little doubt that the mutations in the cyst fluid are derived from the cyst locule walls and indicate that the cyst fluid provides an excellent representation of the neoplastic cells in an IPMN.

EXAMPLE 5

GNAS Mutations in Invasive Cancers Associated with IPMNs

Prior whole exome sequencing had not revealed any GNAS mutations in 24 typical PDA that occurred in the absence of an associated IPMN (29). We extended these data by examining 95 additional surgically resected PDAs in pancreata without evidence of IPMNs for mutations in GNAS R201H or R201C, using the ligation assay described above. Again, no GNAS mutations were identified in PDAs arising in the absence of IPMNs.

We suspected that IPMNs containing GNAS mutations had the potential to progress to an invasive carcinoma because fluids from IPMNs with high-grade dysplasia contained such mutations (Table 1). However, in light of the multilocular and multifocal nature of IPMNs described above, it was not clear whether the cells of the locule(s) that progress to an invasive carcinoma were those that contained GNAS mutations. To address this question, we purified DNA from invasive pancreatic adenocarcinomas that developed in association with IPMNs. In each case, the neoplastic cells of the IPMN and of the invasive adenocarcinoma were carefully microdissected. In seven of the eight patients, the identical GNAS mutation found in the neoplastic cells of the IPMN was found in the concurrent invasive adenocarcinoma (FIG. **9** (Table S5)). The KRAS mutational status of the PDA was consistent with that of the associated IPMN in the same seven cases. In the eighth case, the KRAS and GNAS mutations identified in the neoplastic cells of the IPMN were not found in the associated PDA, suggesting that this invasive cancer arose from a separate precursor lesion (FIG. **9** (Table S5)). Though KRAS mutations were found commonly in both types of PDAs, there was a dramatic difference between the prevalence of GNAS mutations in PDAs associated with IPMNs (7 of 8) vs. that in PDAs unassociated with IPMNs (0 of 116; p<0.001, Fisher's Exact Test).

EXAMPLE 6

A Protocol for Enrichment on Beads

Cleave Oligos from the Chip

Place the chip into the corner of a Micro-Seal bag (Model 50068, DAZEY corporation) cut to ~10.5×5.5 cm.

Seal the unsealed two sides so that the bag ends up 8 cm×2.6 cm, tightly wrapping the chip.

While in the Seal-a-Meal bag, treat for five hours with 3 ml 28% ammonium hydroxide at room temperate by rotator (360 deg rotation). (Make sure the chip is fully immersed in the solution)

Transfer the solution into two 2-ml eppendorf tubes, and speed vacuum dried at temperate 50° C. (normally it will take 5-8 hours)

(For speed vacuum, turn on the cooler one hour before you use the vacuum)

Re-dissolve the oligos in a combined 400 ul RNase and DNase free water.

Amplify the Oligos

Make 3×50 ul PCR mix for each chip, the PCR mix contains the following:

X ul H2O

1 ul (well 1), 2 ul (well 2), 5 ul (well 3)

2 ul forward primer (25 uM): 5'-TGATCCCGCGACGA*C-3', where * indicates phosphorothioate

2 ul reverse primer (25 uM): 5'-GACCGCGACTCCAG*C-3', where * indicates phosphorothioate

4 ul MgCl2 (50 mM)

5 ul 10× Platinum Taq buffer (Life Technologies)

4 ul dNTPs (10 mM each)

1 ul Platinum Taq (5 U/ul, Life Technologies) (Titanium and Phusion both did not work).

Note: Because of the alkalic condition after cleavage, the more template you add, the less PCR product you get.

The cycling conditions were: 1×98° C. for 30 s

35 cycles of 98° C. for 30 s, 40° C. for 30 s, 60° C. for 15 s, 72° C. for 45 s

one cycle of 72° C. for 5 min

Run the gel to see a smear from 60 bp to 120 bp. 120 bp product may be dimers, which won't interfere with capture.

The PCR products were combined, and add 2 ul Sodium Acetate (3M, pH 5.2) purified using a MinElute Purification Column (Qiagen), elute twice in 65° C. pre-warmed buffer with 17 ul each (total of 34 ul).

End-Repair the PCR Product

End-repair using End-IT DNA End-Repair Kit (Epicentre) as follows:

34 ul DNA

5 ul 10× End-Repair Buffer

5 ul dNTP Mix

5 ul ATP

1 ul End-Repair Enzyme Mix

Incubate at room temperature for 45 minutes,

Purified using a MinElute Purification Column (Qiagen), elute twice in 65° C. pre-warmed buffer with 17.5 ul each (total of 35 ul).

Ligate the PCR Product

The PCR products were ligated to form concatamers using the following protocol:

35 ul End-Repaired DNA product

40 ul 2×T4 DNA ligase buffer (Enzymatics Inc.)

5 ul T4 DNA ligase (600 units/ul; Enzymatics Inc.)

The mix was incubated at room temperature for at least 4 hours, (you can leave it overnight.)

The product was purified using QiaQuick PCR Purification Column (Qiagen) (not MinElute), elute twice in 65° C. pre-warmed buffer with 25 ul each (total of 50 ul).

Quantify by absorption at 260 nm. (Normally you get around 3 ug DNA product.)

Dilute the product to 20 ng/ul using TE buffer.

Isothermal Amplification of the Probe with Bio-dUTP [RepliG-Midi Kit (not Mini Kit), Qiagen]

TABLE 1

Preparation of Buffer D1 (Volumes given are suitable for up to 15 reactions)

Component	Volume
Reconstituted Buffer DLB	9 µl
Nuclease-free water	32 µl
Total volume	41 µl

TABLE 2

Preparation of Buffer N1 (Volumes given are suitable for up to 15 reactions)

Component	Volume
Stop solution	12 μl
Nuclease-free water	68 μl
Total volume	80 μl

Place 2.5 μl template DNA into a microcentrifuge tube.

Add 2.5 μl Buffer D1 to the DNA. Mix by vortexing and centrifuge briefly

Incubate the samples at room temperature (15-25° C.) for 3 min.

Add 5 μl Buffer N1 to the samples. Mix by vortexing and centrifuge briefly.

Prepare a master mix on ice according to Table 3 (see below). Mix and centrifuge briefly.

Important: Add the master mix components in the order listed in Table 3. After addition of water and REPLI-g Midi Reaction Buffer,

briefly vortex and centrifuge the mixture before addition of REPLI-g Midi DNA Polymerase. The master mix should be kept on ice and used

immediately upon addition of the REPLI-g Midi DNA Polymerase.

TABLE 3

Preparation of Master Mix

Component	Volume/reaction
REPLI-g Midi Reaction Buffer	14.5 μl
Biotin-dUTP(1 mM) (Cat. No.11093070910, Roche Applied Science)	2.5 ul
REPLI-g Midi DNA Polymerase	0.5 μl
Total volume	17.5 μl

Add 17.5 μl of the master mix to 10 μl denatured DNA that was neutralized with N1 as described above. Transfer the mix to the PCR plate.

Incubate at 30° C. for 16 h in PCR machine.

Inactivate REPLI-g Midi DNA Polymerase by heating the sample at 65° C. for 3 min.

Transfer the mix using 2×120 ul TE to a 1.5 ml tube.

Incubate the tube in 100° C. heating block for 20 minutes.

Purify the product using two QiaQuick PCR Purification Columns (Qiagen) (not MinElute), i.e., use 2 columns for one 27.5 ul reaction.

Elute each column twice with 65° C. pre-warmed buffer with 27.5 ul, for a total of 55 ul, so there will be 110 ul of eluate from the two columns which should be pooled.

Quantify by absorption at 260 nm using nanodrop (I know it's single-strand DNA now, but I still use ds-DNA calculations in nanodrop)

In general, you will get ~180-210 ng/ul. If it's too off, there must be something wrong.

DNA Capture

A mix was prepared as follows:

4 ug DNA library (20 ul, 200 ng/ul)

7 ul Human cot-1 DNA (Cat. No. 15279011, Invitrogen)

3 ul Herring Sperm DNA (Cat. No. 15634-017, Invitrogen)

10 ul Blocking Oligos, 1 nmol/ul each.

Block Oligo 1:

AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTC

T

Block Oligo 2:

CAAGCAGAAGACGGCATACGAGATCGGTCTCGGCATTCCTGCTGAACCG

C

5 ul Capture Probe (~200 ng/ul)

The mix is heated at 95° C. for 7 min, and 65° C. for 2 min (use only one compress pad in PCR machine)

Add 25 ul of the 65° C. prewarmed 2.8× hybridization buffer (final conc of hyb buffer will then be 1×)

2.8× hybridization buffer: (14×SSPE, 14×Denhardt's, 14 mM EDTA, 0.28% SDS), using the following reagents:

20×SSPE: (0810-4L, AMRESCO)

Denhardt's Solution, 50×, 50 ml (70468, usb)

EDTA: 0.5M, PH 8.0 (46-034-CI, Mediatech Inc.)

(In case the DNA library cone is <200 ng/ul, then still use 4 ug DNA and 7 ul Cot-1, 3 ul Herring sperm, etc. but use proportionally larger volumes of 2.8× HybBuffer

Incubate at 65 deg for 22 hours for hybridization with PCR machine lid heat on.

Washing Procedure

Wash 50 ul MyOne beads (Invitrogen) 3 times in 1.5 ml tule and resuspend in 60 μl 1× binding buffer (1M NaCl, 10 mM Tris-HCl, pH 7.5, and 1 mM EDTA.)

Add equal volume (70 ul) of 2× binding buffer (2 M NaCl, 20 mM Tris-HCl, pH 7.5, and 2 mM EDTA.) to hybrid mix, and transfer to tube with beads. Total Volume should be 200 ul.

Vortex the mix thoroughly. And rotate 360 deg. for 1 hour at Room Temperature.

After binding, the beads are pulled down, and washed 15 minutes at RT in 0.5 ml Wash Buffer 1 (1×SSC/0.1% SDS)

Wash the beads for 15 minutes at 65° C. on a heating block with shaking, five times in 0.5 ml Wash Buffer 3 (0.1×SSC and 0.1% SDS)

Hybrid-selected DNA are resuspended in 50 μl 0.1 M NaOH at RT for 10 min.

The beads are pulled down, the supernatant transferred to a tube containing 70 μl Neutralizing Buffer (1 M Tris-HCl, pH 7.5)

Neutralized DNA is desalted and concentrated on a QIAquick MinElute column and eluted in 20 μl.

Note: Wash Buffer 2 (5.2 M Betaine, 0.1×SSC and 0.1% SDS) is a more stringent wash buffer.

For more stringent wash, you can substitute the first WB3 wash with WB2, then continue with four washes with WB3.

Change the post-Capture amplification Cycle number to 16 cycles if you use a more stringent wash.

Post-Capture Amplification

PCR mix containing:

20 captured DNA

51 ul dH2O

20 ul 5× Phusion buffer

5 ul DMSO

2 ul 10 mM dNTPs

0.5 ul (50 pmol) Illumina forward primer (QC1 primer for barcoding)

0.5 ul (50 pmol) Illumina reverse primer (Barcoding reverse primers for barcoding)

1 ul Hotstart Phusion enzyme (New England Biolabs)

The cycling conditions were: 1×98° C. for 30 s

14 cycles of 98° C. for 25 s, 65° C. for 30 s, 72° C. for 30 s

one cycle of 72° C. for 5 min

The PCR is done in two wells for each sample, 50 ul each (no oil on top).

The amplified PCR product was purified using a Nucleo-Spin column (Macherey Nagel, inc.), eluted twice in 65° C. pre-warmed buffer with 17.5 ul (total of 35 ul).

Use NanoDrop to quantify yield, which should be ~20 ng/ul.

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SEQUENCE LISTING

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tgatcccgcg acgac 15

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gaccgcgact ccagc 15

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<210> SEQ ID NO 5
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<212> TYPE: DNA
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tccacctgga acttggtctc 20

<210> SEQ ID NO 6
<211> LENGTH: 26
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
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gatcatattc gtccacaaaa tgattc 26

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tgactgaata taaacttgtg gtagttg 27

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<212> TYPE: DNA
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atggagaact tgacgtcctg ttcgctgccg 30

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ttcgctgcca 10

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<213> ORGANISM: Artificial Sequence
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cttcgctgct 10

<210> SEQ ID NO 13
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gtgtcctgac ttggtgtcca ctagtcatgc tt 32

<210> SEQ ID NO 14
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atggagaact tgacgtcctc ctacgccac 29

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<210> SEQ ID NO 16
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ctacgccacg 10

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agctccaact accacggtgt ccactagtca tgctt 35

<210> SEQ ID NO 20
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atggagaact tgacgtcctc ctacgccac 29

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cctacgccaa 10

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cagctccaac taggtgtcca ctagtcatgc tt 32

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<223> OTHER INFORMATION: Probe

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ctgaaacaaa attgaggt 18

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aggacacggc agcga 15

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aggacacagc agcga 15

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<400> SEQUENCE: 26

ctgaaacaaa attgaggt 18

<210> SEQ ID NO 27
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caggacacgg cagcg 15

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caggacatgg cagcg 15

<210> SEQ ID NO 29
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ggagctggtg gcgta 15

<210> SEQ ID NO 31
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<400> SEQUENCE: 31

ggagctgatg gcgta 15

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tgacgataca gctaattca 19

<210> SEQ ID NO 33
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<400> SEQUENCE: 33

ggagctggtg gcgta 15

<210> SEQ ID NO 34
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<400> SEQUENCE: 34

ggagctgttg gcgta 15

<210> SEQ ID NO 35
<211> LENGTH: 19
<212> TYPE: DNA
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<400> SEQUENCE: 35

tgacgataca gctaattca 19

<210> SEQ ID NO 36
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<223> OTHER INFORMATION: Probe

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<400> SEQUENCE: 36

tggagctggt ggcgt 15

<210> SEQ ID NO 37
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<400> SEQUENCE: 37

tggagctcgt ggcgt 15

<210> SEQ ID NO 38
<211> LENGTH: 60
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
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<400> SEQUENCE: 38

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<210> SEQ ID NO 39
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: probes

<400> SEQUENCE: 39

tcccgcgacg accagtgtct gacttcgaca acgccaaggg cctcaacggc tggagtcgcg 60

<210> SEQ ID NO 40
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: probes

<400> SEQUENCE: 40

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<210> SEQ ID NO 41
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: probes

<400> SEQUENCE: 41

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<210> SEQ ID NO 42
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<212> TYPE: DNA
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<220> FEATURE:
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<400> SEQUENCE: 42

tcccgcgacg acacttcgac aacgccaagg gcctcaacgt gaagcactgc tggagtcgcg 60

<210> SEQ ID NO 43
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
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tcccgcgacg actcgacaac gccaagggcc tcaacgtgaa gcactacagc tggagtcgcg 60

<210> SEQ ID NO 44
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<212> TYPE: DNA
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<400> SEQUENCE: 44

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<210> SEQ ID NO 45
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<212> TYPE: DNA
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<210> SEQ ID NO 46
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: probes

<400> SEQUENCE: 46

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<210> SEQ ID NO 47
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<212> TYPE: DNA
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<220> FEATURE:
<223> OTHER INFORMATION: probes

<400> SEQUENCE: 47

tcccgcgacg acagggcctc aacgtgaagc actacaagat ccgcaagcgc tggagtcgcg 60

<210> SEQ ID NO 48
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: probes

<400> SEQUENCE: 48

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<210> SEQ ID NO 49
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<212> TYPE: DNA
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<220> FEATURE:
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<400> SEQUENCE: 49

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<210> SEQ ID NO 50
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<212> TYPE: DNA
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<220> FEATURE:
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<400> SEQUENCE: 50

tcccgcgacg acacgtgaag cactacaaga tccgcaagct ggacagcggc tggagtcgcg 60

<210> SEQ ID NO 51
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<212> TYPE: DNA
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<220> FEATURE:
<223> OTHER INFORMATION: probes

<400> SEQUENCE: 51

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<210> SEQ ID NO 52
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<212> TYPE: DNA
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<400> SEQUENCE: 52

tcccgcgacg acagcactac aagatccgca agctggacag cggcggctgc tggagtcgcg 60

<210> SEQ ID NO 53
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: probes

<400> SEQUENCE: 53

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<210> SEQ ID NO 54
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: probes

<400> SEQUENCE: 54

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<210> SEQ ID NO 55
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: probes

<400> SEQUENCE: 55

tcccgcgacg acagatccgc aagctggaca gcggcggctt ctacatcagc tggagtcgcg 60

<210> SEQ ID NO 56
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence

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<220> FEATURE:
<223> OTHER INFORMATION: probes

<400> SEQUENCE: 56

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<210> SEQ ID NO 57
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: probes

<400> SEQUENCE: 57

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<210> SEQ ID NO 58
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: probes

<400> SEQUENCE: 58

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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: probes

<400> SEQUENCE: 59

tcccgcgacg actggacagc ggcggcttct acatcacctc ccgcacccgc tggagtcgcg 60

<210> SEQ ID NO 60
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: probes

<400> SEQUENCE: 60

tcccgcgacg acacagcggc ggcttctaca tcacctcccg cacccagtgc tggagtcgcg 60

<210> SEQ ID NO 61
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: probes

<400> SEQUENCE: 61

tcccgcgacg acgcggcggc ttctacatca cctcccgcac ccagttcagc tggagtcgcg 60

<210> SEQ ID NO 62
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: probes

<400> SEQUENCE: 62

tcccgcgacg acgcggcttc tacatcacct cccgcaccca gttcaacagc tggagtcgcg 60

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<210> SEQ ID NO 63
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: probes

<400> SEQUENCE: 63

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<210> SEQ ID NO 64
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: probes

<400> SEQUENCE: 64

tcccgcgacg actctacatc acctcccgca cccagttcaa cagcctgcgc tggagtcgcg 60

<210> SEQ ID NO 65
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: probes

<400> SEQUENCE: 65

tcccgcgacg acacatcacc tcccgcaccc agttcaacag cctgcagcgc tggagtcgcg 60

<210> SEQ ID NO 66
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: probes

<400> SEQUENCE: 66

tcccgcgacg actcacctcc cgcacccagt tcaacagcct gcagcagcgc tggagtcgcg 60

<210> SEQ ID NO 67
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: probes

<400> SEQUENCE: 67

tcccgcgacg accctcccgc acccagttca acagcctgca gcagctgggc tggagtcgcg 60

<210> SEQ ID NO 68
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: probes

<400> SEQUENCE: 68

tcccgcgacg accccgcacc cagttcaaca gcctgcagca gctggtgggc tggagtcgcg 60

<210> SEQ ID NO 69
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<212> TYPE: DNA
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<220> FEATURE:
<223> OTHER INFORMATION: probes

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<400> SEQUENCE: 69

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<210> SEQ ID NO 70
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: probes

<400> SEQUENCE: 70

tcccgcgacg accgtgtcct gacttctgga atctttgaga ccaagttcgc tggagtcgcg 60

<210> SEQ ID NO 71
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: probes

<400> SEQUENCE: 71

tcccgcgacg acgtcctgac ttctggaatc tttgagacca agttccaggc tggagtcgcg 60

<210> SEQ ID NO 72
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: probes

<400> SEQUENCE: 72

tcccgcgacg acctgacttc tggaatcttt gagaccaagt tccaggtggc tggagtcgcg 60

<210> SEQ ID NO 73
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: probes

<400> SEQUENCE: 73

tcccgcgacg acacttctgg aatctttgag accaagttcc aggtggacgc tggagtcgcg 60

<210> SEQ ID NO 74
<211> LENGTH: 60
<212> TYPE: DNA
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<220> FEATURE:
<223> OTHER INFORMATION: probes

<400> SEQUENCE: 74

tcccgcgacg acggcctgtt gcgtctcagc agcagtgcac taaataacgc tggagtcgcg 60

<210> SEQ ID NO 75
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
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<400> SEQUENCE: 75

tcccgcgacg acctgttgcg tctcagcagc agtgcactaa ataacgaggc tggagtcgcg 60

<210> SEQ ID NO 76

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<211> LENGTH: 60
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<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: probes

<400> SEQUENCE: 76

tcccgcgacg acttgcgtct cagcagcagt gcactaaata acgagtttgc tggagtcgcg 60

<210> SEQ ID NO 77
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: probes

<400> SEQUENCE: 77

tcccgcgacg accgtctcag cagcagtgca ctaaataacg agtttttgc tggagtcgcg 60

<210> SEQ ID NO 78
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<212> TYPE: DNA
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<220> FEATURE:
<223> OTHER INFORMATION: probes

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tcccgcgacg acctcagcag cagtgcacta aataacgagt tttttaccgc tggagtcgcg 60

<210> SEQ ID NO 79
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
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<400> SEQUENCE: 79

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<210> SEQ ID NO 80
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: probes

<400> SEQUENCE: 80

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<210> SEQ ID NO 81
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
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<400> SEQUENCE: 81

tcccgcgacg acagtgcact aaataacgag ttttttaccc atgcggctgc tggagtcgcg 60

<210> SEQ ID NO 82
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<212> TYPE: DNA
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<220> FEATURE:
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<400> SEQUENCE: 82

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tcccgcgacg acgcactaaa taacgagttt tttacccatg cggctcaggc tggagtcgcg 60

<210> SEQ ID NO 83
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: probes

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tcccgcgacg acctaaataa cgagtttttt acccatgcgg ctcagagcgc tggagtcgcg 60

<210> SEQ ID NO 84
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
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tcccgcgacg acaataacga gttttttacc catgcggctc agagctgggc tggagtcgcg 60

<210> SEQ ID NO 85
<211> LENGTH: 60
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: probes

<400> SEQUENCE: 85

tcccgcgacg acaacgagtt ttttacccat gcggctcaga gctggcgggc tggagtcgcg 60

<210> SEQ ID NO 86
<211> LENGTH: 60
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: probes

<400> SEQUENCE: 86

tcccgcgacg acgagttttt tacccatgcg gctcagagct ggcgggaggc tggagtcgcg 60

<210> SEQ ID NO 87
<211> LENGTH: 60
<212> TYPE: DNA
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<210> SEQ ID NO 88
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<213> ORGANISM: Artificial Sequence
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tcccgcgacg acccaccagg gtggccatca aaaccctgaa gcctggcagc tggagtcgcg 60

<210> SEQ ID NO 89
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<213> ORGANISM: Artificial Sequence
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<210> SEQ ID NO 90
<211> LENGTH: 96
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
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tcccgcgacg acgggtggcc atcaaaaccc tgaagcctgg cacgatgtgc tggagtcgcg 60
tctcagtgtc tgacttcgac aacgccaagg gcctca 96

<210> SEQ ID NO 91
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<212> TYPE: DNA
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cagtgtctga cttcgacaac gccaagggcc tcaacg 36

<210> SEQ ID NO 92
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tgtctgactt cgacaacgcc aagggcctca acgtga 36

<210> SEQ ID NO 93
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ctgacttcga caacgccaag ggcctcaacg tgaagc 36

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acttcgacaa cgccaagggc ctcaacgtga agcact 36

<210> SEQ ID NO 95
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<212> TYPE: DNA
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tcgacaacgc caagggcctc aacgtgaagc actaca 36

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<400> SEQUENCE: 96

acaacgccaa gggcctcaac gtgaagcact acaaga 36

<210> SEQ ID NO 97
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acgccaaggg cctcaacgtg aagcactaca agatcc 36

<210> SEQ ID NO 98
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<212> TYPE: DNA
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<400> SEQUENCE: 98

ccaagggcct caacgtgaag cactacaaga tccgca 36

<210> SEQ ID NO 99
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<212> TYPE: DNA
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agggcctcaa cgtgaagcac tacaagatcc gcaagc 36

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tcaacgtgaa gcactacaag atccgcaagc tggaca 36

<210> SEQ ID NO 102
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<213> ORGANISM: Artificial Sequence
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<400> SEQUENCE: 102

acgtgaagca ctacaagatc cgcaagctgg acagcg 36

<210> SEQ ID NO 103
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<212> TYPE: DNA
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tgaagcacta caagatccgc aagctggaca gcggcg 36

<210> SEQ ID NO 104
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<212> TYPE: DNA
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<400> SEQUENCE: 104

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<210> SEQ ID NO 105
<211> LENGTH: 36
<212> TYPE: DNA
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actacaagat ccgcaagctg gacagcggcg gcttct 36

<210> SEQ ID NO 106
<211> LENGTH: 36
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
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<400> SEQUENCE: 106

acaagatccg caagctggac agcggcggct tctaca 36

<210> SEQ ID NO 107
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<212> TYPE: DNA
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<220> FEATURE:
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<400> SEQUENCE: 107

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<210> SEQ ID NO 108
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<212> TYPE: DNA
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<220> FEATURE:
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<400> SEQUENCE: 108

tccgcaagct ggacagcggc ggcttctaca tcacct 36

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<210> SEQ ID NO 109
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gcaagctgga cagcggcggc ttctacatca cctccc 36

<210> SEQ ID NO 110
<211> LENGTH: 36
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
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<400> SEQUENCE: 110

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<210> SEQ ID NO 111
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: probes

<400> SEQUENCE: 111

tggacagcgg cggcttctac atcacctccc gcaccc 36

<210> SEQ ID NO 112
<211> LENGTH: 36
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: probes

<400> SEQUENCE: 112

acagcggcgg cttctacatc acctcccgca cccagt 36

<210> SEQ ID NO 113
<211> LENGTH: 36
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: probes

<400> SEQUENCE: 113

gcggcggctt ctacatcacc tcccgcaccc agttca 36

<210> SEQ ID NO 114
<211> LENGTH: 36
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: probes

<400> SEQUENCE: 114

gcggcttcta catcacctcc cgcacccagt tcaaca 36

<210> SEQ ID NO 115
<211> LENGTH: 36
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:

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<223> OTHER INFORMATION: probes

<400> SEQUENCE: 115

gcttctacat cacctcccgc acccagttca acagcc 36

<210> SEQ ID NO 116
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: probes

<400> SEQUENCE: 116

tctacatcac ctcccgcacc cagttcaaca gcctgc 36

<210> SEQ ID NO 117
<211> LENGTH: 36
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
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<400> SEQUENCE: 117

acatcacctc ccgcacccag ttcaacagcc tgcagc 36

<210> SEQ ID NO 118
<211> LENGTH: 36
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: probes

<400> SEQUENCE: 118

tcacctcccg cacccagttc aacagcctgc agcagc 36

<210> SEQ ID NO 119
<211> LENGTH: 36
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: probes

<400> SEQUENCE: 119

cctcccgcac ccagttcaac agcctgcagc agctgg 36

<210> SEQ ID NO 120
<211> LENGTH: 36
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: probes

<400> SEQUENCE: 120

cccgcaccca gttcaacagc ctgcagcagc tggtgg 36

<210> SEQ ID NO 121
<211> LENGTH: 36
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: probes

<400> SEQUENCE: 121

tgccgtgtcc tgacttctgg aatctttgag accaag 36

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<210> SEQ ID NO 122
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: probes

<400> SEQUENCE: 122

cgtgtcctga cttctggaat ctttgagacc aagttc 36

<210> SEQ ID NO 123
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: probes

<400> SEQUENCE: 123

gtcctgactt ctggaatctt tgagaccaag ttccag 36

<210> SEQ ID NO 124
<211> LENGTH: 36
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: probes

<400> SEQUENCE: 124

ctgacttctg gaatctttga gaccaagttc caggtg 36

<210> SEQ ID NO 125
<211> LENGTH: 36
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: probes

<400> SEQUENCE: 125

acttctggaa tctttgagac caagttccag gtggac 36

<210> SEQ ID NO 126
<211> LENGTH: 36
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: probes

<400> SEQUENCE: 126

ggcctgttgc gtctcagcag cagtgcacta aataac 36

<210> SEQ ID NO 127
<211> LENGTH: 36
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: probes

<400> SEQUENCE: 127

ctgttgcgtc tcagcagcag tgcactaaat aacgag 36

<210> SEQ ID NO 128
<211> LENGTH: 36
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: probes

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<400> SEQUENCE: 128

ttgcgtctca gcagcagtgc actaaataac gagttt 36

<210> SEQ ID NO 129
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: probes

<400> SEQUENCE: 129

cgtctcagca gcagtgcact aaataacgag tttttt 36

<210> SEQ ID NO 130
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: probes

<400> SEQUENCE: 130

ctcagcagca gtgcactaaa taacgagttt tttacc 36

<210> SEQ ID NO 131
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: probes

<400> SEQUENCE: 131

agcagcagtg cactaaataa cgagtttttt acccat 36

<210> SEQ ID NO 132
<211> LENGTH: 36
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: probes

<400> SEQUENCE: 132

agcagtgcac taaataacga gttttttacc catgcg 36

<210> SEQ ID NO 133
<211> LENGTH: 36
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: probes

<400> SEQUENCE: 133

agtgcactaa ataacgagtt ttttacccat gcggct 36

<210> SEQ ID NO 134
<211> LENGTH: 36
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: probes

<400> SEQUENCE: 134

gcactaaata acgagttttt tacccatgcg gctcag 36

<210> SEQ ID NO 135
<211> LENGTH: 36

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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: probes

<400> SEQUENCE: 135

ctaaataacg agttttttac ccatgcggct cagagc 36

<210> SEQ ID NO 136
<211> LENGTH: 36
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: probes

<400> SEQUENCE: 136

aataacgagt tttttaccca tgcggctcag agctgg 36

<210> SEQ ID NO 137
<211> LENGTH: 36
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: probes

<400> SEQUENCE: 137

aacgagtttt ttacccatgc ggctcagagc tggcgg 36

<210> SEQ ID NO 138
<211> LENGTH: 36
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: probes

<400> SEQUENCE: 138

gagtttttta cccatgcggc tcagagctgg cgggag 36

<210> SEQ ID NO 139
<211> LENGTH: 36
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: probes

<400> SEQUENCE: 139

gtaccaccag ggtggccatc aaaaccctga agcctg 36

<210> SEQ ID NO 140
<211> LENGTH: 36
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: probes

<400> SEQUENCE: 140

ccaccagggt ggccatcaaa accctgaagc ctggca 36

<210> SEQ ID NO 141
<211> LENGTH: 36
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: probes

<400> SEQUENCE: 141

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ccagggtggc catcaaaacc ctgaagcctg gcacga 36

<210> SEQ ID NO 142
<211> LENGTH: 96
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: probes

<400> SEQUENCE: 142

gggtggccat caaaaccctg aagcctggca cgatgtgtgc ctactgcctc tcagtgtctg 60

acttcgacaa cgccaagggc ctcaacgtga agcact 96

<210> SEQ ID NO 143
<211> LENGTH: 60
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: probes

<400> SEQUENCE: 143

cctactgcct ctcagtgtct gacttcgaca acgccaaggg cctcaacgtg aagcactaca 60

<210> SEQ ID NO 144
<211> LENGTH: 60
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: probes

<400> SEQUENCE: 144

actgcctctc agtgtctgac ttcgacaacg ccaagggcct caacgtgaag cactacaaga 60

<210> SEQ ID NO 145
<211> LENGTH: 60
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: probes

<400> SEQUENCE: 145

gcctctcagt gtctgacttc gacaacgcca agggcctcaa cgtgaagcac tacaagatcc 60

<210> SEQ ID NO 146
<211> LENGTH: 60
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: probes

<400> SEQUENCE: 146

tctcagtgtc tgacttcgac aacgccaagg gcctcaacgt gaagcactac aagatccgca 60

<210> SEQ ID NO 147
<211> LENGTH: 60
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: probes

<400> SEQUENCE: 147

cagtgtctga cttcgacaac gccaagggcc tcaacgtgaa gcactacaag atccgcaagc 60

<210> SEQ ID NO 148
<211> LENGTH: 60

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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: probes

<400> SEQUENCE: 148

tgtctgactt cgacaacgcc aagggcctca acgtgaagca ctacaagatc cgcaagctgg 60

<210> SEQ ID NO 149
<211> LENGTH: 60
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: probes

<400> SEQUENCE: 149

ctgacttcga caacgccaag ggcctcaacg tgaagcacta caagatccgc aagctggaca 60

<210> SEQ ID NO 150
<211> LENGTH: 60
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: probes

<400> SEQUENCE: 150

acttcgacaa cgccaagggc ctcaacgtga agcactacaa gatccgcaag ctggacagcg 60

<210> SEQ ID NO 151
<211> LENGTH: 60
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: probes

<400> SEQUENCE: 151

tcgacaacgc caagggcctc aacgtgaagc actacaagat ccgcaagctg gacagcggcg 60

<210> SEQ ID NO 152
<211> LENGTH: 60
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: probes

<400> SEQUENCE: 152

acaacgccaa gggcctcaac gtgaagcact acaagatccg caagctggac agcggcggct 60

<210> SEQ ID NO 153
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: probes

<400> SEQUENCE: 153

acgccaaggg cctcaacgtg aagcactaca agatccgcaa gctggacagc ggcggcttct 60

<210> SEQ ID NO 154
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: probes

<400> SEQUENCE: 154

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ccaagggcct caacgtgaag cactacaaga tccgcaagct ggacagcggc ggcttctaca 60

<210> SEQ ID NO 155
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: probes

<400> SEQUENCE: 155

agggcctcaa cgtgaagcac tacaagatcc gcaagctgga cagcggcggc ttctacatca 60

<210> SEQ ID NO 156
<211> LENGTH: 60
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: probes

<400> SEQUENCE: 156

gcctcaacgt gaagcactac aagatccgca agctggacag cggcggcttc tacatcacct 60

<210> SEQ ID NO 157
<211> LENGTH: 60
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: probes

<400> SEQUENCE: 157

tcaacgtgaa gcactacaag atccgcaagc tggacagcgg cggcttctac atcacctccc 60

<210> SEQ ID NO 158
<211> LENGTH: 60
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: probes

<400> SEQUENCE: 158

acgtgaagca ctacaagatc cgcaagctgg acagcggcgg cttctacatc acctcccgca 60

<210> SEQ ID NO 159
<211> LENGTH: 60
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: probes

<400> SEQUENCE: 159

tgaagcacta caagatccgc aagctggaca gcggcggctt ctacatcacc tcccgcaccc 60

<210> SEQ ID NO 160
<211> LENGTH: 60
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: probes

<400> SEQUENCE: 160

agcactacaa gatccgcaag ctggacagcg gcggcttcta catcacctcc cgcacccagt 60

<210> SEQ ID NO 161
<211> LENGTH: 60
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence

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<220> FEATURE:
<223> OTHER INFORMATION: probes

<400> SEQUENCE: 161

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<210> SEQ ID NO 162
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: probes

<400> SEQUENCE: 162

acaagatccg caagctggac agcggcggct tctacatcac ctcccgcacc cagttcaaca 60

<210> SEQ ID NO 163
<211> LENGTH: 60
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: probes

<400> SEQUENCE: 163

agatccgcaa gctggacagc ggcggcttct acatcacctc ccgcacccag ttcaacagcc 60

<210> SEQ ID NO 164
<211> LENGTH: 60
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: probes

<400> SEQUENCE: 164

tccgcaagct ggacagcggc ggcttctaca tcacctcccg cacccagttc aacagcctgc 60

<210> SEQ ID NO 165
<211> LENGTH: 60
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: probes

<400> SEQUENCE: 165

gcaagctgga cagcggcggc ttctacatca cctcccgcac ccagttcaac agcctgcagc 60

<210> SEQ ID NO 166
<211> LENGTH: 60
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: probes

<400> SEQUENCE: 166

agctggacag cggcggcttc tacatcacct cccgcaccca gttcaacagc ctgcagcagc 60

<210> SEQ ID NO 167
<211> LENGTH: 60
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: probes

<400> SEQUENCE: 167

tggacagcgg cggcttctac atcacctccc gcacccagtt caacagcctg cagcagctgg 60

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<210> SEQ ID NO 168
<211> LENGTH: 60
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: probes

<400> SEQUENCE: 168

acagcggcgg cttctacatc acctcccgca cccagttcaa cagcctgcag cagctggtgg 60

<210> SEQ ID NO 169
<211> LENGTH: 60
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: probes

<400> SEQUENCE: 169

gcggcggctt ctacatcacc tcccgcaccc agttcaacag cctgcagcag ctggtggcct 60

<210> SEQ ID NO 170
<211> LENGTH: 60
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: probes

<400> SEQUENCE: 170

gcggcttcta catcacctcc cgcacccagt tcaacagcct gcagcagctg gtggcctact 60

<210> SEQ ID NO 171
<211> LENGTH: 60
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
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<400> SEQUENCE: 171

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<210> SEQ ID NO 174
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<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: probes

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<400> SEQUENCE: 174

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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
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<212> TYPE: DNA
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<220> FEATURE:
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: probes

<400> SEQUENCE: 177

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<210> SEQ ID NO 178
<211> LENGTH: 60
<212> TYPE: DNA
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<220> FEATURE:
<223> OTHER INFORMATION: probes

<400> SEQUENCE: 178

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<210> SEQ ID NO 179
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: probes

<400> SEQUENCE: 179

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<210> SEQ ID NO 180
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
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<400> SEQUENCE: 180

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<210> SEQ ID NO 181

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<211> LENGTH: 60
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: probes

<400> SEQUENCE: 181

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<210> SEQ ID NO 182
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<212> TYPE: DNA
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<210> SEQ ID NO 183
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<210> SEQ ID NO 186
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<212> TYPE: DNA
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<210> SEQ ID NO 187
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gcactaaata acgagttttt tacccatgcg gctcagagct ggcgggagcg cctggctgat 60

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ggacctggaa cggtaccacc agggtggcca tcaaaaccct gaagcctggc acgatgtctc 60

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<400> SEQUENCE: 193

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<210> SEQ ID NO 194
<211> LENGTH: 60
<212> TYPE: DNA

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<213> ORGANISM: Artificial Sequence
<220> FEATURE:
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<400> SEQUENCE: 194

acggtaccac cagggtggcc atcaaaaccc tgaagcctgg cacgatgtct ccagaggcct 60

The invention claimed is:

1. A method for enriching a sample comprising nucleic acids for target nucleic acid analytes, comprising the steps of:

synthesizing a set of probes for one or more analytes of interest, wherein the probes are complementary to plus or minus strands of a target nucleic acid analyte, wherein each probe has a 5' universal priming site and a 3' universal priming site;

amplifying the set of probes using primers complementary to the 5' universal priming site and primers complementary to the 3' universal priming site;

ligating the amplified probes to form double-stranded concatamers;

isothermally amplifying the double-stranded concatamers to form single-stranded amplified concatamers;

binding the single-stranded amplified concatamers to a solid support;

contacting the solid support with the sample comprising nucleic acids under hybridization conditions so that complementary nucleic acids in the sample are captured on the solid support and non-complementary nucleic acids are removed;

eluting captured nucleic acids from the solid support.

2. A method for enriching a sample for target nucleic acid analytes, comprising the steps of:

synthesizing a set of probes for one or more analytes of interest, wherein the probes are complementary to plus or minus strands of a target nucleic acid analyte, wherein each probe has a 5' universal priming site and a 3' universal priming site;

amplifying the set of probes using primers complementary to the 5' universal priming site and primers complementary to the 3' universal priming site;

ligating the amplified probes to form double-stranded concatamers;

isothermally amplifying the double-stranded concatamers in the presence of biotinylated nucleotides, such that biotinylated nucleotides are incorporated into amplified, single-stranded concatamers;

contacting the amplified, single-stranded concatamers with the sample nucleic acids to form a mixture, and subjecting the mixture to hybridization conditions so that complementary nucleic acids in the mixture hybridize to the amplified, single-stranded concatamers;

contacting the mixture with a solid support so that hybridized nucleic acids are captured on the solid support, wherein the solid support comprises avidin or streptavidin;

washing the solid support to remove nucleic acids which do not comprise biotin;

eluting captured nucleic acids from the solid support.

3. The method of claim **1** further comprising the step of: analyzing nucleotide sequence of the eluted, captured nucleic acids.

4. The method of claim **3** wherein the analyzing is massively parallel.

5. The method of claim **2** further comprising: analyzing nucleotide sequence of the eluted, captured nucleic acids.

6. The method of claim **5** wherein the analyzing is massively parallel.

7. The method of claim **1** further comprising the step of: amplifying the eluted, captured nucleic acids.

8. The method of claim **2** further comprising the step of: amplifying the eluted, captured nucleic acids.

9. The method of claim **1** wherein the set of probes are synthesized on a solid array.

10. The method of claim **9** wherein the set of probes are cleaved from the solid array to form an aqueous solution comprising the set of probes.

11. The method of claim **2** wherein the set of probes are synthesized on a solid array.

12. The method of claim **11** wherein the set of probes are cleaved from the solid array to form an aqueous solution comprising the set of probes.

13. The method of claim **1** wherein the probes are complementary to both plus and minus strands of a target nucleic acid analyte.

14. The method of claim **1** wherein probes for one analyte overlap with a register of three nucleotide displacement.

15. The method of claim **13** wherein probes for one analyte overlap with a register of three nucleotide displacement.

16. The method of claim **1** wherein the solid support is a filter.

17. The method of claim **1** wherein the solid support is a bead.

18. The method of claim **2** wherein the probes are complementary to both plus and minus strands of a target nucleic acid analyte.

19. The method of claim **2** wherein probes for one analyte overlap with a register of three nucleotide displacement.

20. The method of claim **18** wherein probes for one analyte overlap with a register of three nucleotide displacement.

21. The method of claim **2** wherein the solid support is a filter.

22. The method of claim **2** wherein the solid support is a bead.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 9,670,527 B2
APPLICATION NO. : 14/130007
DATED : June 6, 2017
INVENTOR(S) : Bert Vogelstein et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

In the Specification

Column 1 Lines 4-7 (government support statement) please replace with the following:

STATEMENT OF GOVERNMENTAL INTEREST
This invention was made with government support under grant numbers CA043460, CA057345, CA062924, awarded by the National Institutes of Health. The government has certain rights in the invention.

Signed and Sealed this
Sixth Day of February, 2018

Joseph Matal

Joseph Matal
Performing the Functions and Duties of the
Under Secretary of Commerce for Intellectual Property and
Director of the United States Patent and Trademark Office

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 9,670,527 B2
APPLICATION NO. : 14/130007
DATED : June 6, 2017
INVENTOR(S) : Bert Vogelstein et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

On the Title Page

Item (54) and in the Specification, Column 1 Line 2 in the Title, delete, "COMPLIMENTARY" and add --COMPLEMENTARY--, therefore.

Signed and Sealed this
Twenty-eighth Day of January, 2020

Andrei Iancu
Director of the United States Patent and Trademark Office